INTERACTIONS BETWEEN COVER CROP RESIDUE AND MICROBIAL COMMUNITY AFFECT WEED SUPPRESSION

BY

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DISSERTATION

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ABSTRACT

The environmental and health risks from the use of herbicides have led to interest in developing alternative methods for agricultural weed control. Incorporation of cover crop residues is an important approach that contributes to alternative weed management systems. The cover crop residue-associated allelochemicals can inhibit weed germination, growth and production. Soil microbial communities are especially important for the allelochemical potential of a cover crop residue, because microorganisms can modify residues and allelochemicals to make them more or less phytotoxic. In addition to their effects on allelochemicals, soil microbial communities can also directly suppress weeds. To improve weed biocontrol, it is critical to understand how soil microorganisms interact with cover crop residues to suppress weed.

The goal of this dissertation is to evaluate the contributions of microbes, cover crop residues, and their interactions to weed suppression. The first experiment examined the temporal dynamics of weed suppression over time to determine the microbial suppression, residue suppression, and their interactions. Results show that microbial activity directly suppressed weed germination and seedling growth, but also indirectly helped weeds by degrading cover crop-derived allelochemicals. These results suggest that the weed suppression shifted from a predominantly chemical phase to a predominantly microbial phase. To identify putative weed suppressive microbes, the microbial communities associated with diseased and stunted seedlings were characterized, and organisms present were examined for desirable weed suppressive traits. The identified putative weed suppressive microbes can be considered as a starting point for future selection of biocontrol agents.

To determine the effects of management practices on weed suppression, I investigated the interactions between microbial communities and cover crop residues in three agricultural management systems (tillage, no tillage and organic system). Overall, the organic and tillage management systems offered higher microbial and cover crop-derived weed suppression than the no-tillage system. Different microbial communities were associated with dead seeds and diseased seedlings. These microbes also differed among the three agricultural management systems. This result indicates the potential of managing the soil microbiome for desirable weed suppression.

The cover crop associated allelochemicals negatively affected microbial attacks to seedlings. However, the allelochemicals also induced seedling leakage that made seedling more susceptible to microbial attacks by triggering microbial chemotactic behaviors.

Understanding how soil microbial communities interact with cover crop residues in agricultural systems has important implications for the weed biocontrol strategies. This dissertation suggests that naturally occurring soil microbial communities can be used and managed for sustainable weed control in agricultural systems.

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CHAPTER1: INTRODUCTION

Sustainable weed management

Currently, herbicide use is the primary method for weed management in industrialized countries and is intensifying in developing countries (Liebman et al., 2001; Sigurd, 2003). However, the use of herbicides also brings some unintended impacts, such as herbicide-resistant weeds (Ervin et al., 2014), surface and ground water contamination (Thurman et al., 1991), and off-target threats to wild animal and human health (Sotherton et al., 1985). Such problems have been recognized for several decades in the management of weeds. These problems led scientists to develop the concept of integrated weed management (IWM) in the 1960s and 1970s (Bantilan et al., 1974; Harwood et al., 1974). Emphasis of IWM is placed on modifying the habitat characteristics of weeds to reduce weed densities, promote crop growth, and conserve and promote natural enemy organisms that attack weeds (Liebman et al., 2001; Walker et al., 1982; Watson et al., 1989).

How can transitions be made from conventional weed management systems toward more sustainable, ecologically based systems? Walker (1982) and McWhorter (1982) have proposed a systems-level approach to manipulate multiple ecological interactions in agroecosystems to prevent weed problems. One of these ecological approaches is including cover crops in crop rotations. Cover crops can suppress weeds through various mechanisms, including resource and light competitions (Liebman et al., 1993; Teasdale, 1996), interference with weed life cycles (Moyer et al., 2000), physical suppression by cover crop residues (Moore et al., 1994), and release of phytotoxic chemicals (Kruidhof et al., 2009; Samedani et al., 2013; Teasdale et al., 2012). Another important ecological approach is promoting soil pathogens to suppress weed recruitment, growth, and reproduction (Chee-Sanford et al., 2006; Kremer et al., 1996). Soil microbial communities respond to the incorporation of cover crops (Baumann et al., 2009; Carrera et al., 2007). Both plant beneficial and detrimental microbes can be stimulated, which influences the overall soil suppression on weeds. This reciprocal influence between cover crop residues and microbial communities has the potential to be used for optimizing weed control effects. However, this optimization is mainly restricted by the lack of basic

knowledge on the ecology of weed suppressive microorganisms. In order to effectively utilize ecological biocontrol, it is important to understand the ecological interactions between the soil microbial community and cover crop residues on weed suppression and to characterize the key microbial antagonists of weeds and their responses to agricultural management practices.

Weed management through negative plant-soil feedback

Plant-soil-microbe feedbacks have been influential mechanisms of plant dynamics in natural, unmanaged systems (Bever, 2003; J. N. Klironomos, 2002). Plant-soil feedback explains many ecological phenomena, such as plant species coexistence, plant succession, plant invasion and establishment in new environments. Negative plant-soil feedback from soil-borne microbial antagonists is ubiquitous in the natural environment (Johnson et al., 2012; J. Klironomos, 2002; Mangan et al., 2010). Soil microbial communities can generate negative feedback to plants in both direct and indirect ways. Direct negative feedback can be the result of accumulation of microbial antagonists, such as host-specific bacterial and fungal pathogens (Mangan et al., 2010; Mitchell et al., 2003). Indirect negative feedback can be the result of different specificity of microbes to co-occurring plant species. This can lead to the counter-intuitive observation that negative feedback is possible even when plants are associated with mutualistic microbes (Bever et al., 1997). For example, arbuscular mycorrhizal (AM) fungi can benefit one plant species more than its neighboring plant, which negatively affects the relative fitness of the neighboring plant (Bever, 2002a, 2002b).

Use of a direct feedback pathway is not straightforward for weed biocontrol, because farmers don't allow weeds to reach the high densities that are necessary to build up negative plant-soil feedback. However, farmers might use the soil microbes as a means for weeds and crops to interact with each other indirectly. This means the creation of soil communities that are adverse to weeds but not to cash crops. For example, incorporation of a cover crop could derive microbial communities that are harmful to weeds. The diseased seedlings (A. Conklin, 1999) and abundance of some soil pathogens (Bonanomi et al., 2011) (Rothrock et al., 1995) increase following incorporation of cover crop residues. The soil pathogens may directly infect weed species (Burdon et al., 1981;

Renwick et al., 1990), or produce phytotoxic compounds to stress or kill weeds (Robert E Hoagland, 1990; R. E. Hoagland, 2001). Additionally, indirect weed suppression can be generated by benefiting crops more than weeds. A crop cultivar that is susceptible to mycorrhizal colonization experiences benefits from the mycorrhizal symbiosis, but some non-host weed species can be negatively affected by the mycorrhizal fungi. This antagonistic effects of mycorrhizal fungi on non-host weeds may reduce competitive effects of non-host weeds (Jordan et al., 2000; Koide et al., 1992). Thus, it may be possible to manipulate the soil mycorrhizal fungal community so that it is more favorable to the crop than the weeds. These examples demonstrate the potential to influence the performances of weeds relative to crops through changing soil microbial communities.

Although plant-soil feedback theory provides potential for weed control, microbial biocontrol agents remain largely unexploited for weed management (Charudattan, 2001; Liebman et al., 2001). An important reason is the lack of understanding of the basic biology and ecology of pathogen–weed interactions (Liebman et al., 1997; Liebman et al., 2001). Attention to these issues may improve the development and practical application of weed biocontrol. First, microbial biocontrol agents are often viewed as stress factors that are most useful when they are integrated with various other weed management strategies (Kennedy et al., 1996), such as incorporation of cover crop residues. In order to promote this integration, studies are needed to understand the interaction between them. Second, plant-soil feedback can depend on site-specific differences in abiotic and biotic soil properties. For example, tillage and cover crop residue management are particularly important for regulating microbe-plant interactions (Drijber et al., 2000; Grunwald et al., 2000; Spedding et al., 2004). Such variance in soil management may make interpretations and predictions of weed control much more challenging. Thus, more studies are needed to determine how weed suppression changes under different types of management in crop systems.

Allelochemical weed suppression through cover crop residues

Cover crops that contain a high level of phytotoxic allelochemicals are well suited for residue-mediated weed suppression (Macias et al., 2007; Singh et al., 2003). The visible effects of allelopathy are frequently observed as inhibited or delayed seed

germination, reducing seedling growth but increasing seedling anomalies (Barnes et al., 1986; Kruidhof et al., 2009; Ohno et al., 2000). A variety of secondary plant metabolites have been implicated as possible allelopathic compounds. Of these, the most commonly proved allelochemical agents are phenolic compounds (Inderjit, 1996; Jilani et al., 2008). The total phenolic content of water extracts of plant residues is general positively correlated with the allelopathic suppressive potential (Ismail et al., 2002; Ohno et al., 2001).

Allelochemicals produced by cover crop residues in the soil are dynamic during the residue decomposition process. Plant residues in soil undergo several physical, chemical and biological processes (Hadas et al., 2004; Parr et al., 1978). The changes of these factors over time alter the compositions and quantities of allelochemicals, which can further influence the allelopathic effects on plants (An et al., 2000; Bonanomi et al., 2006). Generally, allelochemical weed suppression is transient, with the greatest effect noted immediately after incorporation and gradually declining over a period of roughly two weeks (A. E. Conklin et al., 2002; Mohler et al., 2012).

Allelopathic effects may also vary depending on the soil management method. Allelopathic effects of sorghum residue have been shown to reduce the yields of wheat in no-tilled fields but have no effect in tilled fields (Roth et al., 2000). The phenolic compounds are more toxic to plants in soils of low fertility than well-fertilized soil (Stowe et al., 1980). The transitory nature of allelopathy and its dependence on soil conditions represent significant challenges to the use of residue-derived allelochemicals for weed management. Thus, it is important to understand the dynamics of allelochemicals in soil in order to take best advantage of their effective time window (An et al., 2000; Inderjit et al., 2005). Second, research is also needed to determine the effects of soil management on allelochemical weed suppression.

In agriculture, green manures are plant residues that are incorporated into soil through tillage. Allelochemicals released from green manures can effectively suppress weed germination, establishment, and growth (A. E. Conklin et al., 2002; Liebman et al., 2006; Ohno et al., 2000). Red clover is a good green manure cover crop (Stopes et al., 1996). Among 19 grassland species, the aqueous extract of clover shoot material is the most inhibitory to other plant species (Scott, 1975). Phenolic compounds, especially

isoflavones, are highly abundant in red clover materials (Krenn et al., 2002; Saviranta et al., 2010). These phenolic compounds are suggested to be the major sources of allelopathic activities of red clover residues (A. E. Conklin et al., 2002; Liebman et al., 2002; Ohno et al., 2000; Ohno et al., 2001). The two main isoflavones in red clover are biochanin A and formononetin, and other isoflavones from red clover include daidzin, daidzein, genistin, genistein, pratensein, prunetin, and calycosin (Saviranta et al., 2010; Tsao et al., 2006). Most weed suppressive studies of red clover used total phenolic content to represent all phenolic compounds (Liebman et al., 2006; Ohno et al., 2000; Ohno et al., 2001). However, different phenolic compounds in the mixture have various functions (Inderjit, 1996), which may not be consistent with the effects of total phenolic content. Therefore, the relative importance of individual compounds in suppression has not been recognized yet. To better utilize the allelochemical weed suppression, we need to characterize the qualities and quantities of these allelochemicals produced by red clover residues, and also the inhibitory effect of individual compounds.

Weed suppression through soil microbes

It has been known by scientists for a long time that some microbial groups, such as deleterious rhizobacteria (DRB) (Nehl et al., 1997; Suslow et al., 1982) and soil-borne pathogens (Baker, 1968; Renwick et al., 1990), have adverse effects on plant growth and development. Soil microbes need to target the seeds and seedlings of host plants to attack. But little is known about the initiation of the targeting processes. One hypothesis is that seeds and seedlings may provide a significant source of carbon or nitrogen nutrition for soil microorganisms (Inderjit et al., 2008; Nelson, 2004). For the microbial communities that are closely attached to seeds or seedlings, plant-derived carbon exudates have strong direct effects on microbial reorganization and colonization (Chen et al., 2012; Inderjit et al., 2008). For microbial communities in the soil zones surrounding seeds and seedlings, chemicals released by the plants can be signals that provide a direction for soil microbes' movement towards seed and seedling (Barbour et al., 1991; Klerks et al., 2007).

It may be "counter-intuitive," but natural born soil antagonists can be exploited for their positive effects on agricultural production. Classic selection of biocontrol agents uses cultivation and screening approaches to find weed antagonistic microbial strains.

Some of them have shown suppressive effects in the field, such as inhibition of weed germination, growth and competitive abilities with crops (Kremer, 1993; Kremer et al., 1996; TeBeest, 1996). However, most studies to date have only dealt with well-known microbial groups. These known weed control agents may only present a small portion of the whole microbial weed suppressive community. Soil microbiology is a challenging area that is far from fully describing microbial community diversity, composition, interaction, and function. The culturable microorganisms constitute only 1% of real microbial populations (Ward et al., 1990). Furthermore, microbes may only demonstrate pathogenicity under suitable conditions. For example, a saprobe may convert to a pathogen when a host plant is immune deficient or otherwise under stress (Dunn et al., 2006; Mycock et al., 1992). Moreover, the negative effects of microbes on plants are not limited to direct pathogenic attacks on seeds and seedlings. Indirect negative effects can also play an important role in plant growth and development; examples include competition with seedlings for nutrients (Kumar et al., 2008; Schimel et al., 1989) and benefiting competitor plants more than host plants (Bever, 2003; Westover et al., 2001). It is possible that many weed suppressive microbes have not been discovered by the classic cultural-based approach. The modern high-throughput molecular approach allow us to examine the whole microbial community (Caporaso et al., 2010) and identify novel putative weed suppressive microbes. In the long term, the capability to manipulate individual microbial taxa may improve the potential to manage agricultural soils by promoting these weed suppressive microorganisms.

Interactions of soil microbes and cover crop residue on weed suppression

To manage weeds in cover crop systems, it is important to understand the interactions between soil microbial communities and cover crop residue-derived allelochemicals. Multiple weed biocontrol approaches, such as microbial and allelochemical weed controls, can be complementary to weed suppression (Liebman et al., 2009; Liebman et al., 2001). And also allelochemicals in the soil are always subject to microbial modifications, which substantially changes the allelochemical availability in the soil (Inderjit, 2005; Jilani et al., 2008).

Weed suppression and disease incidence can be increased following plant residue incorporation. For example, live soil amended with cover crop residues reduces seedling germination more than sterilized soil or live soil with no residue additions (Mohler et al., 2012). Lower germination rate, smaller wild mustard seedlings, and more diseased seedlings were found in residue-amended soil than unamended soil (A. E. Conklin et al., 2002). Pathogenic *Pythium* populations are increased in soil with fresh and air-dried residues incorporated (Manici et al., 2004). Alfalfa seedling damping-off caused by *Pythium ultimum* and *R. solani* increases when soils are amended with alfalfa residues (Bonanomi et al., 2011).

Until now, the potential mechanisms for the link between the residue incorporation and the increased microbial weed suppression are still unknown. First, it is possible that residue extracts can delay germination and slow down seedling growth (Liebman et al., 2006), which extends the exposure time of seeds and seedlings to the soil pathogens (Davis et al., 2007). Thus, the possibility of microbial attack can increase. Second, toxic products from residues assist the invasion and attack of pathogens by damaging root tissues and inducing seedling leakage (Chandler et al., 1974; Patrick et al., 1964; Toussoun et al., 1963). Seedlings which were grown in residue extracts developed bigger cankers than seedlings in distilled water (Toussoun et al., 1963). The seedlings that were previously exposed to residue extracts were more susceptible to fungal attacks than seedlings with no exposure to residue extracts (Chandler et al., 1974; Toussoun et al., 1963). Moreover, these seedlings that have been exposed to the extracts exude more sugar and amino acid than unexposed controls (Toussoun et al., 1963). Because microbes demonstrate chemotactic behaviors to root exudates (Broek et al., 1995; Klerks et al., 2007), the allelochemical-induced leakage may stimulate microbial movement towards seedlings.

A complicating issue for the allelochemicals has arisen with recent discoveries that the phytotoxic chemicals produced by plants can inhibit some pathogens (Daayf et al., 2012; Morrissey et al., 1999). Some of the metabolites have both allelopathic effects on plants and antimicrobial properties on pathogens. For example, the main allelochemicals in red clover are a type of phenolic compounds – isoflavones (Ohno et al., 2000; Ohno et al., 2001). Isoflavones have been identified as part of anti-microbial molecules known as

phytoalexins (Nicholson et al., 1992; Reynolds et al., 2003). One important function of isoflavone is defending against pathogenic attack. Daidzein inhibits the growth of the pathogen *Fusarium culmorum*, and glycitein and formononetin can reduce mycelial development in *Aspergillus ochraceus* (Kramer et al., 1984). So, clearly, allelochemicals can act as precursors of pathogen infection and also pathogen inhibitors, which can assist or hinder pathogenic attacks to seeds and seedlings. These findings indicate the important roles of some allelochemicals in influencing soil microbe-weed interactions.

Another important microbe-residue interaction that influences the residue derived weed suppression is the microbial transformation of allelochemicals. Allelochemicals in the soil are subjected to various physicochemical and biological processes. Allelochemicals may be transformed into less or more toxic forms by soil microorganisms, or they may serve as a carbon skeleton for new compounds (Blum et al., 2000; Jilani et al., 2008). Microbial degradation of allelochemicals is commonly found in soil, which reduces the total allelopathic activity (Furbo et al., 2011; Jilani et al., 2008; Kaur et al., 2009). For example, soil microbes can metabolize phenolic acids within a couple of hours (Blum et al., 2000). The allelopathy of a compound, which is detected in sterile condition, is significantly diminished when non-sterile soil is used (Ito et al., 1998; Kaur et al., 2009; Perry et al., 2007).

Microbial responses to agricultural management

The efficacy of allelochemical and microbial biocontrols has been doubted because of inconsistent results obtained from site to site and from year to year (Harper, 1977; Wardle et al., 1998). A possible reason is that we overlook the importance of local heterogeneity of soil microbial communities. In an agricultural system, the microbial communities are greatly impacted by agricultural management (Gary D Bending et al., 2004; Doran, 1980). Reduced and zero tillage practices reduce soil erosion, protect soil organic matter (Havlin et al., 1990; Reganold et al., 1987), and also enhance soil microbial diversity and biomass (Drijber et al., 2000; Kandeler et al., 1999) in comparison with conventional tillage. On the contrary, tillage destroys the diversity of soil microsites together with the assemblages of soil microorganisms that live on them. This damage could result in a reduction in both the composition and functional diversity

of the soil microbial community (Beare et al., 1995; Sparling et al., 1997). Organic farmers manage for high soil organic matter, which is correlated with high soil enzyme activities (Moeskops et al., 2010). Furthermore, use of synthetic pesticides can negatively impact soil biota (Miller et al., 1998; Pampulha et al., 2006). As a consequence, microbial activity and diversity are often higher in soil of organic farms than conventional farms (G. D. Bending et al., 2004; Carpenter-Boggs et al., 2000). Microbial communities are also different between organic and conventional farming systems (Bossio et al., 1998; Mader et al., 2002).

Because microbial communities can be changed by agricultural management practices, it is possible to manage soil functional microbial communities to deliver a better weed suppressive soil. To achieve this goal, we should have a better understanding of allelochemical-soil microbial interactions and how they change under different agricultural management.

Overview of dissertation

The presence of plant deleterious microbes in the natural environment suggests that the soil microbial communities may be managed as an untapped but valuable natural resource for weed control. Identification of putative weed suppressive microbes and their interactions with green manure provides foundational knowledge for strategies that can turn this natural resource into an agent of effective weed control.

Microbial communities and green manures both have weed suppressive potential. But their individual and interaction effects have not been fully understood so far (An et al., 2001; Bonanomi et al., 2006). In Chapter 2, I conducted a time series experiment to investigate the effects of microbial communities, water soluble and insoluble compounds of red clover residues on the germination and seedling growth of wild mustard. The main objective is to understand the dynamic changes of microbe- and allelochemical-induced weed suppression after red clover residue incorporation. I hypothesize that the microbial community and the residue fractions will alter the temporal dynamics of residue-derived allelochemicals in the soil, and that these changes will in turn modify the duration of the weed suppressive window.

The results of Chapter 2 suggest that microbial communities have two important weed suppressive activities: seedling growth inhibition and seedling disease promotion. The overall aim of Chapter 3 is to identify putative microbial taxa with weed suppressive potential using samples obtained from Chapter 2. I used high-throughput sequencing to characterize the whole bacterial and fungal community on diseased seedlings and in their rhizosphere soil. I proposed that useful weed control microorganisms will possess one or more of the following desirable traits: 1) they should be correlated with diseased and stunted seedlings; 2) they can be enriched by the addition of green manures; and 3) they are keystone players in the microbial network.

Agricultural management practices can strongly influence the soil microbial community (Carrera et al., 2007; Feng et al., 2003; Kandeler et al., 1999), which may lead to different microbe- and allelochemical-induced weed suppression. To extend the single field study in Chapter 2 to a broader context, in Chapter 4, I aimed to understand the changes of weed suppressive potential across a range of management regimes. I examined microbial and red clover residue weed suppression and soil microbial communities under three agricultural managements: tillage, no tillage and organic farming. I hypothesized that the long-term agricultural management practices would have significant and predictable effects on the soil microbial community. The different microbial communities would lead to different microbe- and allelochemical-induced weed suppression, but suppression would be relatively consistent within the same management.

The isoflavones in red clover may have both negative and positive effects on microbial attacks on weeds. The antimicrobial features of some isoflavones can inhibit microbial pathogenicity (Reynolds et al., 2003). However, these chemical compounds can also assist microbial attacks on seedlings by damaging root tissues, destabilizing root membranes and inducing seedling leakage (Chandler et al., 1974; Patrick et al., 1964; Toussoun et al., 1963). Because microbes demonstrate chemotactic behaviors (Begonia et al., 1994; Broek et al., 1995), the seedling leakage can be chemical cues to attract soil pathogens (Begonia et al., 1994; Toussoun et al., 1963). In Chapter 5, I first experimentally manipulated the allelochemical levels in the soil to test the phytotoxic effects of allelochemicals on seedling disease incidence. Then, I examined the effects of

allelochemicals on the composition of seedling exudates. Finally, I investigated microbial chemotactic movement to the seedling exudates and characterized chemotactic microbes. My hypothesis is that allelochemicals would inhibit microbial attacks on seedlings, but they would also stimulate seedling leakage, which would serve as chemoattractants for some soil microbes.

Together these four studies present a novel contribution to studies of allelochemical and microbial weed suppression. I identified a list of putative weed suppressive microbes warranting further study and demonstrated the potential for use of naturally occurring, plant-suppressive microbes as a novel weed suppressive approach.

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CHAPTER 2: INTERACTIONS BETWEEN ALLELOCHEMICALS AND THE MICROBIAL COMMUNITY AFFECT WEED SUPPRESSION FOLLOWING COVER CROP RESIDUE INCORPORATION INTO SOIL[1]

Abstract

Background and Aims: The objective of this study is to understand how soil microorganisms interact with cover crop-derived allelochemicals to suppress weed germination and growth following cover crop residue incorporation.

Methods: I conducted a time series experiment crossing sterilized and non-sterilized soil with four different residue treatments. I measured weed seed germination rates, radicle elongation, disease incidence, and cover crop-derived phenols in seed germination bioassays. I partitioned the total weed suppression into three sources: microbial only inhibition, residue only inhibition, and the microbe-residue interaction.

Results: Microbial activity suppressed weed germination and growth for 30 days, while cover crop-derived allelochemicals provided suppression for a limited time. There was an antagonistic interaction between microbes and allelochemicals. This interaction was strongest for water-soluble allelochemicals, while residue fractions containing intact plant tissues retained greater suppressiveness even in the presence of a live microbial community.

Conclusions: Microbial activity can directly suppress weed germination and growth, but microorganisms also indirectly help weeds by degrading cover crop-derived allelochemicals. As a result of these interactions, cover crop-derived weed suppression in agricultural soils shifts from an early allelochemical-dominated phase to a later phase where microbial suppression is more important.

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Introduction

Because widespread herbicide use in agriculture leads to environmental damage and increased emergence of herbicide-resistant weeds, there is much interest in alternative forms of weed control (Charudattan, 2001; Inderjit et al., 2005; Liebman et al., 2009; Weston, 1996). Rotations involving cover crops are one of these alternatives (Liebman et al., 2000; Liebman et al., 2009; Wortman et al., 2013). Cover crops can suppress weeds through resource and light competition (Liebman et al., 1993; Teasdale, 1996), disruption of weed life cycles (Moyer et al., 2000), physical suppression by cover crop residues (Moore et al., 1994) and release of phytotoxic chemicals associated with cover crop residues (Kruidhof et al., 2009; Samedani et al., 2013; Teasdale et al., 2012). Cover crop residue-associated allelochemicals can suppress weed seed germination (Seigler, 1996), seedling establishment (Singh et al., 2003; Weston, 1996) and weed growth rates (Mirsky et al., 2011; Wardle, 1995).

The total allelochemical potential of a cover crop residue is a combination of water-soluble phytotoxins released by residues prior to decomposition, as well as the insoluble phytotoxins released by microorganisms during decomposition (J.P. Barnes et al., 1986; Harper et al., 1982) and the subsequent microbial transformation of theses phytotoxins (J.P. Barnes et al., 1986; Inderjit, 2005). Microbes can detoxify water-soluble allelochemicals released soon after cover crop residue incorporation (Jilani et al., 2008), but they can also transform harmless plant-derived compounds to more toxic forms (Williamson et al., 1992). Microbes play important roles in releasing additional allelochemicals bound up in the recalcitrant fractions of cover crop residues (J. P. Barnes et al., 1987). These insoluble allelochemicals can constitute a significant fraction of total allelopathic potential of a cover crop residue (Harper et al., 1982), so microbes can slowly release residue-derived allelochemicals, extending the longevity of a cover crop's effectiveness.

In addition to their effects on allelochemicals, soil-borne microbial antagonists can provide biological forms of weed suppression. Various pathogenic strains with weed control potential have been isolated from soil or infected weed seedlings (Kremer, 1993). Pathogenic infection of weeds can reduce weed germination rates and retard the growth of seedlings (Davis et al., 2007). Cover crop residues may enhance soil pathogen growth

(Conklin et al., 2002; Mohler et al., 2012) and potentially encourage pathogens to attack damaged weed seedlings (Chandler et al., 1974; Z. Patrick et al., 1964). Mohler and colleagues recently showed that unsterilized "live" soil (i.e. with a natural microbial community) reduces seedling germination rates when cover crop residues are incorporated, and the combined effect of residues and live microorganisms is greater than the effect of either of these components alone (Mohler et al., 2012). Exposure to cover crop-derived allelochemicals increases the density of fungal lesions on plant seedlings (Toussoun et al., 1963). Furthermore, disease incidence on seedlings and the abundance of soil-borne pathogens can both be increased with cover crop residue addition to soils (Conklin et al., 2002; Rothrock et al., 1995). Thus, it is reasonable to suspect that disease-causing microorganisms may provide a leverage point for more effective weed suppression potential.

The dynamics of cover crop- and microorganism-derived weed suppression will depend on the rate of allelochemical release, the properties of the allelochemicals, and the activities of allelochemical-degrading and seedling-infecting soil microorganisms. These factors will interact to create a "window" of weed-suppression potential. Residue-induced suppression of a sensitive plant will take place only when there is an overlap in time between the period of sensitivity of the plant and the window of the suppression potential (Kruidhof et al., 2009). In order for allelochemical weed suppression to confer a competitive advantage to cash crops (Kruidhof et al., 2009), cover crop residues must be managed to maximize the suppressive effects on target weeds and also to avoid phytotoxicity on cash crops. These management decisions should be based on the dynamics of allelochemicals and soil microorganisms, and thus there is a need to understand how these chemical and microbial factors interact and change following residue incorporation. Here I characterize the weed-suppressive capacity of different soluble and insoluble fractions of cover crop residues. I examine the temporal dynamics of weed-suppression over time in the presence and absence of a live soil community to understand the differing roles of allelochemicals and soil microorganisms and how these roles change over time. I hypothesize that the presence of a live microbial community will alter the temporal dynamics of cover crop-derived allelochemicals in the soil, and that these changes will in turn modify the duration of the weed suppressive window.

Methods and materials

Preparation of red clover residue fractions and soil treatments

For my model cover crop, I selected Mammoth red clover (*Trifolium pratense*), a widely used legume cover crop with high allelochemical potential (Liebman et al., 2006a). I planted red clover in April 2013 at the Maxwell Trust site of the c Urbana, IL in a field plot that had been maintained in a corn-soybean rotation for over 20 years. The soil at the field was a Catlin silt loam (Oxyaquic Argiudoll) with the following characteristics: 7% sand, 68% silt, 25% clay, 4.2% soil organic carbon, pH 7.2. I harvested red clover plants at the bud stage after 14 weeks of growth.

I processed the red clover plants in order to evaluate the weed-suppressive potential of three different residue-derived fractions: 1) the water-soluble fraction, 2) the insoluble faction (i.e. bound in the "straw"), and 3) the fresh residue fraction (which contained both the soluble and insoluble components). The fresh residue fraction was intended to mimic additions of red clover residues in a typical "green manure" management strategy, while the water-soluble and straw fractions were intended to allow us to tease out the separate contributions of soluble and insoluble allelochemicals (Creamer et al., 1996). Plants destined for the fresh residue treatments were stored at 4 ℃ for no more than 1 week prior to use in the bioassays described below. The other plants were freeze-dried to facilitate the extraction of water-soluble allelochemicals. I used freeze drying because it can best preserve the original forms of isoflavones in red clover as compared to oven drying (Tsao et al., 2006). I cut 20 g of the freeze-dried plants into 5-cm pieces and shook them in 400 ml sterilized, deionized water for 16 h at 23 ℃. I used cheese-cloth to recover the large pieces of residue, and then we centrifuged (4000g, 10 min) the liquid fraction to further remove particulate matter. I concentrated the liquid fraction, containing the readily available, water-soluble chemicals, five-fold by freeze-drying the extract to a final volume of 80 ml. I stored the resulting concentrated extract at -20 ℃ for use in the bioassays described below. The large red clover pieces recovered from the cheese-cloth were re-dried in a freeze dryer and stored at 4 ℃ until their use in bioassays described below. I refer to these water-extracted residues as the "straw" fraction, which have been leached of readily available water-soluble chemicals.

I collected soil for the bioassays in June 2013 from the same field where red clover was planted, to a depth of 10 cm. I collected bulk soil samples and stored them in a sealed plastic bucket at 4 ℃ for up to 1 month before use. I divided this soil into two portions, one of which was triple-autoclaved at 120 ℃ for 1 h to kill soil microorganisms.

Experimental design

I combined microbial treatments ("live" vs. sterilized soil) and red clover fractions (fresh residues, water-soluble extracts, straw fraction, and water-only controls) in a fully-factorial experiment. I constructed 480 mesocosms in Magenta vessels (GA-7 vessels (77 mm × 77 mm × 97 mm) from Sigma-Aldrich Co. St. Louis, MO) to represent the 2 microbial x 4 residue fraction x 6 time point (see below) combinations (10 replicate mesocosms, each). Fresh red clover and straw fractions were completely dispersed in a Zip-lock bag and sterilized by UV light for 2h on each side prior to addition to the mesocosms in order to control for the introduction of microorganisms present on the plant tissue (Wilson et al., 1999). I filter-sterilized extracts through a 0.22-micron filter prior to addition to mesocosms. Because 2% red clover was sufficient to elicit a germination response in mustard seeds (Liebman et al., 2006b) and was similar to a field incorporation rates of red clover (Dyck et al., 1995), I added 2% (by weight) fresh red clover residues, or the equivalent amount of potentially bioactive compounds, to each mesocosm. Each mesocosm contained 110 g of soil. Therefore, I added (110 g x 2% =) 2.2 g of fresh red clover residue cut into 5-cm pieces to all fresh residue treatment mesocosms. The freeze-drying procedure used to produce the water-soluble extract and straw residue fractions (see above) resulted in a 6-fold reduction in the mass of straw residues in comparison to the fresh litter. Therefore, I added (2.2 g x 6 =) 13.2 g of straw residues to all straw residue treatment mesocosms. I added the equivalent amount of water-soluble extracts found in 2.2 g of fresh residue to our water-soluble extracts treatment mesocosms; I had extracted 20 g of fresh residues into a final volume of 80 ml water (see above), and thus I added (2.2 g residue x 80 ml / 20 g residue =) 8.8 ml of extract. Finally, I added 8.8 ml of double-distilled water to the water-only treatment mesocosms. Mesocosms were fitted with lids with a filter-covered hole to maintain sterile conditions and minimize water loss.

All mesocosms were set up on the same day, but I assayed their weed-suppression potential at different times in order to understand how weed suppression changes with time after residue incorporation. I conducted these assays at days 0, 2, 4, 8, 16, and 30. At each of these time points, I randomly selected 10 replicate mesocosms from each of the 8 microbe x residue fraction treatments (80 mesocosms, in all) and used all of the soil in each mesocosm to conduct the bioassays described below. Mesocosms were arranged in a glasshouse according to a fully randomized design, and I re-randomized the placement of the remaining mesocosm after each assay time points.

Bioassays of germination and seedling growth

I used the seed germination bioassay technique of Dabney and colleagues (1996), as described below, to assess the microbial and allelochemicals effects on weed germination and growth at each of the six time points described above. I used IdaGold mustard (*Sinapis alba*) as a model weed because it is a common weed of temperate agroecosystems, and because this variety has a very high, uniform germination rate. Therefore, seed dormancy was unlikely to be a factor in estimation of seed germination. Before the bioassay began, 10g soil were collected into separate centrifuge tubes and stored at -20 ℃ for analysis of soil phenolic carbon content (see below).

Each bioassay unit was constructed from a different, single mesocosm. I placed 15 mustard seeds in a line 10 cm from the top edge a double layer of 25 cm by 38 cm germination paper (Anchor Paper, St. Paul, MN) moistened with 20 ml of sterilized, deionized water. Then I spread the remaining 100 g of soil from a mesocosm in a 12cm wide band, about 6 cm from the top edge of germination paper, to cover the line of seeds. I placed another moistened sheet of germination paper on top of the seeds and soil, and rolled this entire assembly from the short edge to create a cylinder. I wrapped and sealed each cylinder in a plastic Zip-lock bag to maintain soil moisture content throughout the bioassay. I incubated these bioassay units vertically (i.e. with seeds oriented “up” in the upright cylinder) in a Conviron 125-L incubator (Controlled Environments Limited, Manitoba, Canada) for 7 days with a 16 h light:8 h dark cycle (25 ℃ and 20 ℃, respectively).

After 7 days of incubation, I deconstructed each bioassay unit and recorded the number of germinating seeds and the radicle length of all germinated seedlings. I also

recorded the number of seedlings with visible necrotic lesions on the radicle, which I considered to be infected for the purposes of this study.

Soil total phenolics extraction and measurement

Total phenolic compounds are often used as a proxy for plant-derived allelochemicals (Inderjit, 1996; Ohno et al., 2000). I estimated the phenolic content of mesocosm soils at the time that each was used to construct the bioassays (collected upon setup, as described above) using the methods of Levengood and colleagues (2010) with the following modifications. I ground soils with a mortar and pestle, and I transferred 5 g to a 50ml centrifuge tube. I extracted soils twice with 20ml of 25:70:5 acetonitrile:methanol:acetone for 2h with vigorous shaking. Between extractions I centrifuged samples (4000g, 10 min) and retained the supernatant. I combined the supernatants and reduced the volume to approximately 2 mL with nitrogen flow and heating to 37 °C. I used the Folin-Ciocalteu method (Ainsworth et al., 2007) to quantify total soil phenolics from these extracts as follows. I mixed 0.1 mL of extract with 0.2 mL of 1:10 diluted Folin–Ciocalteu's phenol reagent and 0.8 mL of 700nm sodium carbonate and incubated for 2 h at 23 ℃. I then measured absorbance at 765 nm using gallic acid standards to create a standard curve.

HPLC analysis

Isoflavones are the main allelopathic compounds in red clover (Macias et al., 2007). I used HPLC to analyze nine main isoflavones in red clover (Krenn et al., 2002). They are biochanin A, calycosin, daidzein, daidzin, formononetin, genistein, genistin, glycitein, and prunetin. Samples were analyzed with Metabolomics Center's 5500 QTRAP LC/MS/MS system (AB Sciex, Foster City, CA) with a 1200 series HPLC system (Agilent Technologies, Santa Clara, CA) including a degasser, an autosampler, and a binary pump. The LC separation was performed on a Bidentate C18 100A column (2.1 x 150mm, 4μm) (MicroSolv Technology Corp. Eatontown, NJ) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). The flow rate was 0.4 mL/min. The linear gradient was as follows: 0-1min, 90%A; 15min, 80%A; 25-35min, 67%A; 40-42min, 50%A; 42.5-48min, 90%A. The autosampler was set at 5°C. The injection volume was 2 μL. Mass spectra were acquired in the positive

mode with ion spray voltage of 5500 V. The source temperature was 450 ℃. The curtain gas, ion source gas 1, and ion source gas 2 were 35psi, 65psi, and 55psi, respectively. Multiple reaction monitoring (MRM) was used for quantitation: daidzin m/z 417.1 - m/z 255.0, genistin m/z 433.1 - m/z 271.0, daidzein m/z 255.0-m/z 199.0, glycitein m/z 285.0 - m/z 270.1, calycosin m/z 285.0 - m/z 270.1, genistein m/z 271.1 - m/z 153.0, formononetin m/z 269.1 - m/z 213.0, prunetin m/z 285.1 - m/z 242.0, biochanin A m/z 285.1 -m/z 270.0, and internal standard 6-hydroflavone m/z 241.0 - m/z 137.0.

Statistical Analysis

For each 15-seed bioassay unit, I calculated the following values: percentage of germinated seeds, mean radicle length of germinated seeds, and percentage of infected seedlings (based on the number of infected seedlings divided by the number of germinated seedlings). I used ANOVA and Tukey's HSD (honest significant difference) test to analyze main and interactive effects of microbe treatments, residue fraction treatments, and time.

I also sought to quantify the relative contribution of microbes and residues to the weed suppressive effect at each time point in our experiment. I considered two different dimensions of weed suppressiveness of soils: Germination Inhibition (GI) and Radicle Elongation Inhibition (RI). I compared different sets of treatments in order to partition GI and RI into three parts: microbe-only inhibition, residue-only inhibition, and the microbe-by-residue interaction. I estimated microbe-only inhibition by comparing live and sterile soil treatments in mesocosms receiving water-only additions (no residues). I estimated residue-only inhibition by comparing water-only and residue addition treatments in sterile soil (no microbes). I estimated the interaction, which represents non-additive effects due to the combination of live microbes and residues, by subtracting the microbe-only effects and residue-only effects from the total inhibition. To exemplify, the calculations for the various components of GI are as follows:

$$\text{Microbe-only\_GI} = G_{\text{sterile soil + water-only}} - G_{\text{live soil + water-only}}$$

$$\text{Residue-only\_GI} = G_{\text{sterile soil + water-only}} - G_{\text{sterile soil + residue}}$$

$$\text{Interaction\_GI} = G_{\text{sterile soil + water-only}} - G_{\text{live soil + residue}} - \text{Microbe-only\_GI} - \text{Residue-only\_GI}$$

Where G was the mean germination percentage for all replicated units exposed to a particular treatment (i.e. $G_{\text{sterile soil + water-only}}$ was mean germination in sterile soil with distilled water). Note that the first two terms in the Interaction_GI estimate the total inhibition in treatments with live microbes and residues. I calculated RI using mean radicle length in place of G in the formulas above. Values of GI and RI close to 0 indicate no effect on germination or radicle elongation. Positive values indicate inhibition (i.e. lower germination rate or shorter radicle lengths in treatments compared to controls). By looking at relative responses at each time point, I was able to remove any inherent variability due differences in seed biology and the experimental bioassay environment at the different time points, as these differences would also affect the water-only controls.

To explore overall patterns in chemical composition, I used Principal Components Analysis ordination. I also fitted seed germination, radicle length and days after incorporation on chemical composition ordination. All of these analyses used functions from package "vegan" in R (Oksanen et al., 2009).

The importance of individual allelochemicals on germination and growth was evaluated using partial least squares regression (PLSR, also commonly known as Latent Structure Regression (Carrascal et al., 2009) with a variable selection method. I used PLSR to model germination percentage or radicle length as a function of the multivariate HPLC data. Conceptually, PLSR is similar to using a Principal Components Analysis ordination of the HPLC data to construct a set of orthogonal "latent variables" representing variation in chemical composition; these latent variables are then used as independent variables in regressions against a response variable. However, unlike Principal Components Analysis, the ordination in PLSR creates latent variables that maximize the covariance between the chemical composition data and the response variable (germination percentage or radicle length). I used variable importance in projection (VIP) as the variable selection method (Gosselin et al., 2010; Wold et al., 1993). The loading of individual chemical components on the latent variables can be used as a measure of the importance of each chemical component to seed germination or seedling radicle length. Variable importance was estimated by weighting the latent variable loading of each compound by the contribution of its latent variables to weed germination or radicle length. I accumulated the weighted loading of each variable from

each component and considered variable importance larger than 10% as a selection threshold. PLSR was performed in R using function plsr() in the package "pls" (Mevik et al., 2001).

Results

Effects of cover crop residues and microbes on weed suppression

Microbe and residue fraction treatments differed significantly with respect to weed seed germination and radicle length, and the interaction was also significant (ANOVA, all factors have P-value < 0.01). Microbes demonstrated high weed suppression potential even in the absence of any residue fractions. In water-only control treatments, seed germination (Figure 2.1) and radicle length (Figure A.1) were consistently reduced by about 50% in live soil compared to sterilized soil (P-value < 0.05). However, the presence of a live microbial community tended to reduce the suppression potential of red clover residues. For fresh residue treatments, seed germination was always higher in live soil than sterilized soil, although this difference was only significant at the alpha = 0.05 level on day 30 (Figure 2.1b). For soil receiving water-soluble extracts, seed germination was significantly higher (more than 60%) in live soil than sterilized soil on days 2 and 4; after day 16 this relationship was reversed, with a higher sterile soil having a higher germination rate than soil with a live microbial community (Figure 2.1c). For straw residue treatments, seed germination rate was higher in sterilized soil than live soil with the exception of days 2 and 4 (Figure 2.1d). The effects of time and treatments on radicle length were generally similar to what I found for seed germination (Figure A.1).

The relative strengths of microbe-only suppression, residue-only suppression, and their interaction varied dynamically over time and across the different residue fractions (Figure 2.2). Microbe-only inhibition was relatively stable over time (Figure 2.2). The residue-only GI of fresh residues was consistently high over the entire experimental period. In contrast, the residue-only GI of water-soluble extracts and straw residues were high for the first four days of the experiment, and then GI declined to very low levels for both of these fractions. The microbe-by-residue interaction almost always decreased the

GI for all fractions (Figure 2.2). The interaction resulted in a very large reduction in GI for water-soluble extracts and live microbes in the first two and four days of the experiment. In contrast, the interaction term gradually reduced the effects of microbes and fresh residues over the course of the experiment. The temporal and treatment-level patterns of RI were generally very similar to those of GI (Figure A.2), but the microbe-by-residue interaction resulted in stronger reductions to RI than GI.

Microbial disease incidence

Microbial infection of seedlings was a post-germination suppressive force. Across all treatments, infected seedlings were on average 27 mm shorter than uninfected seedlings (P-value <0.001 by t-test). I found microbial infection of seedlings in all treatments, although the percentage of infected seedlings varied across treatments and over time (Figure 2.3). Seedlings were fully infected in live soil with distilled water, but fewer than 50% of seedlings were infected in treatments with straw or fresh residues until day 8. By day 16 the infection rate of seedlings was higher than 80% in all treatments.

Total phenol content and weed suppression

Soil total phenols were negatively correlated with weed germination (Figure. 2.4) and radicle length (Figure A.3). The threshold phenol concentration for complete suppression of seed germination was about 20ng per g soil. For low concentrations of soil phenol, the presence of a live microbial community resulted in lower germination rates than sterile soil with similar phenol concentrations (Figure 2.4).

Allelochemical composition

Different fractions of residues released different kinds of phenolic compounds into the soil (Figures 2.5 and 2.6). Fresh residues and water-soluble extracts contained formononetin as the single dominant compound, while straw residues released roughly equal amount of formononetin, biochainin A and prunetin (Figure 2.6). Each of the phenolic compounds assayed here was negatively correlated to weed germination rate and radicle length. Genistin and daidzin, which are the 7-O-beta-D-glucoside derivatives of genistein and daidzein, were relative high in soil with fresh residues and aqueous

extracts. Genistein, prunetin and biochanin A were relative high in soil with straw residues.

The phenolic composition of all residue fractions converged over time to the water-only composition (Figure 2.5b), which contain undetectable concentrations of phenolics (Figure 2.6). This convergence happened more quickly in the live soil than the in the sterilized soil. Allelochemicals from soil with straw residues increased from day 0 to day 2, and then they decreased more slowly than in any other treatment for the duration of the experiment.

Four chemicals were identified as potentially important weed suppressive agents. Formononetin was the most potent weed suppressive chemical in fresh residues and water-soluble extracts (Figure 2.7). Biochainin A and prunetin were the most suppressive chemicals in the straw fraction. Calycosin was also found to contribute to weed suppression in water-soluble extracts and straw residues (Figure 2.7).

Discussion

Because cover crop allelochemical effects are often transient and modified by soil microbes, it has been challenging to consistently apply cover crop-derived allelochemicals to weed control (Jilani et al., 2008; Macias et al., 2007). Here I have demonstrated that both red clover residues and resident soil microbial communities have high potential to inhibit germination and growth of a common agricultural weed. However, these two sources of weed suppression combine in a non-additive way, such that the combined effects of soil microorganisms and red clover residues were smaller than what would be expected based on their separate contributions (Figures 2.2 and A.2). The relative strength of the microbe-by-residue interaction varied over time and across treatments representing different components of red clover residues. Here I discuss the dynamics of this non-additive microbe and residue combination, calling attention to the specific microbial interactions with cover crop residues and their associated allelochemicals that shape the effectiveness of green manure as a weed control strategy.

Microbial interactions with water-soluble allelochemicals

Previous work on cover crop-associated allelochemicals has focused on the water-soluble components, which can be easily extracted from plant tissue and used in laboratory-based experiments (Liebman et al., 2006a; Ohno et al., 2001). While my results agree with these previous studies that water-soluble extracts contain bioactivity that inhibits seed germination, I found that this bioactivity disappears very rapidly in the presence of a live microbial community (Figure 2.1c). The antagonistic microbe-by-residue interaction was sufficient to completely negate the germination suppression potential of water-soluble extracts by the second day of our experiment, and this strong, negative interaction persisted for at least 30 days (Figure 2.2). The rapid onset of a strongly antagonistic microbe-by-extract effect indicates that a focus on easily extractable chemical components of cover crop residues may overestimate their potential for weed suppression in natural settings with soil microorganisms present, and my results underscore the message of previous researchers that soil microorganisms are understudied but critically important mediators of important exterminators of allelopathic activity (Inderjit, 2005).

This antagonistic microbe-by-extract interaction is likely to be the result of rapid microbial degradation of the water-soluble components of red clover residues (Inderjit et al., 2004; Inderjit et al., 2005). In line with this interpretation, I found that the profile of soil phenolic compounds in treatments with water-soluble extracts and live microbes was almost identical to that of water-only controls by day 2 (Figure 2.5b). In addition, the concentrations of red clover isoflavones in water-soluble extract treatments were lower in the presence of live microbes than in sterilized soils (Figure 2.6). However, it is also possible that the water-soluble extracts negatively affected the soil microbial community, which I found to be naturally capable of germination suppression (Figure 2.1a). Many red clover phenols, particularly isoflavones, have antimicrobial effects (Reynolds et al., 2003) and can inhibit the growth of microbial pathogens (Daayf et al., 2012). I note that the negative microbe-by-extract interaction exceeded the extract-only suppression of seedling growth for much of the experiment (Figure A.2), and this means that at least a portion of the negative microbe-by-extract interaction must have come from a reduction in microbial capacity for seedling growth suppression. I also note that seedlings in water-

only control treatments showed signs of infection throughout the experiment, but pathogenic attack on seedlings was low in all of the extract and residue treatments in the early portions of the experiment (Figure 2.3). This may reflect the antimicrobial nature of residue-derived chemicals, but it may also indicate that residues provided an additional resource for soil microorganisms (Blum et al. 1993), resulting in a lower initial attack rate on emerging seedlings.

Several workers have proposed that allelochemical-induced damage to seedlings can stimulate microbial attack (Chandler et al., 1974; Z. A. Patrick et al., 1964; Toussoun et al., 1963). Mohler and colleagues (Mohler et al., 2012) interpreted lower weed emergence in their live soil versus sterilized soil treatments to be a signature of pathogenic weed suppression. In contrast to this previous work, and in contrast to my hypothesis of synergistic pathogen activity, my results provide no evidence of pathogen stimulation by residues or residue-derived chemicals. These discrepancies may be due to differences in microbial community composition or to methodological differences leading to different soil allelochemical concentrations. My results indicate that microbial weed suppression was most important at the very lowest soil phenol concentrations (Figures 2.4 and A.3), and it is possible that there is a "sweet spot" in allelochemical concentration at which pathogen stimulation is greater than antimicrobial inhibition. Whether or not farmers can manage soils, cover crop residues, and cash crop planting around such a sweet spot is an open question, and its answer may depend on the combined influences of residue chemical composition, the method of residue incorporation, and the biological properties of the soil microbial community.

Microbial interactions with solid residue fractions

In contrast to the rapid loss of bioactivity that I found for water-soluble extracts, I found that fresh red clover residues provided prolonged suppression of weed germination, and the presence of live microorganisms did not diminish this suppression for the first 16 days of the experiment (Figure 2.1b). The combination of fresh red clover residues and a live microbial community represents my most field-relevant treatment, and so the microbe-by-residue interaction (Figure A.2) can shed light on in-field dynamics related to soil chemistry and ecology. I propose that fresh red clover residues served as a reservoir of allelochemicals throughout our experiment, and that these allelochemicals were

released in sufficient quantities to inhibit seed germination and seedling growth over a prolonged period of time. The phenolic profile of soil with fresh residues took 16 days to converge on the water-only controls (Figure 2.5), and the concentrations of isoflavones in the fresh residue treatment equaled or exceeded the concentrations found in the water-soluble extract treatment, even when live microbial communities were present (Figure 2.6). I suggest that the allelochemicals in fresh residues were more protected from microbial degradation than when they were added as extracts, giving fresh red clover residues a longer lasting suppressiveness. I identified formononetin as the most important allelochemical in the fresh residue treatments (Figure 2.7), in agreement with previous work demonstrating the potency of formononetin as a plant growth inhibitor (Liu et al., 2013). I note formononetin was also the most important allelochemical in water soluble extracts, and this suggests that fresh residues and water-soluble extracts have similar chemical modes of action, but the longer lasting nature of the fresh residue effect may be due to the slow release of this chemical over time. Interestingly, the soil concentration of formononetin was higher in the fresh residue plus live microbe treatment than it was in the sterile water-soluble extract treatment on day 8 (Figure 2.6); at this time, the fresh residue treatments (live and sterile) were highly suppressive of seed germination (Figure 2.1b), while the water-soluble extract treatments were losing suppressiveness (Figure 2.1c).

Fresh red clover residues provided almost total germination suppression on their own for the first 8 days of the experiment, leaving no room for any additional or synergistic effects of microorganisms; note that the negative interaction is essentially equal in magnitude to the microbe-only suppression for the first 8 days of the experiment (Figure 2.2). My results agree with previous reports of potent red clover residue effects on wild mustard (Ohno et al., 2000) and common lambsquarters emergence (Dyck et al., 1995). I found some seed germination in fresh residue treatments starting on day 16 (Figure 2.1), by which time the antagonistic microbe-by-residue interaction exceeded the microbe-only effect (Figure. 2.2). I propose that between day 8 and day 16 the rate of microbial degradation of allelochemicals exceeded the rate of release from the residues, resulting in a sufficiently low bioactivity for some seeds to germinate successfully. Other

research suggests that this slow degradation of residue derived allelochemicals may provide some degree of seed toxicity over five weeks (Ohno et al., 2001).

Some of the prolonged suppressiveness of red clover residues may have been due to the presence of relatively insoluble phytotoxic compounds in the solid portions of the residues. Phenolic compounds are found in both free and bound forms in plant tissue (Lin et al., 2000), and many bound phenolic compounds are water insoluble and difficult to extract even with organic solvents (TeBeest, 1996). I found that the addition of straw residues--which had been leached of water-soluble compounds--suppressed seed germination over a prolonged period of time (Figure 2.1), a result that is consistent with previous evidence of "physical" suppression by a number of different leached cover crop residues (Creamer et al., 1996). The chemical profile in soils with straw residues was unlike that of any other treatment (Figure 2.5), including much higher concentrations of biochanin A, calycosin, and prunetin than in other treatments (Figure 2.6) and also higher concentrations of the non-glucoside and less water-soluble (Stancanelli et al., 2007) isoflavones genistein, daidzein, and glycitein. Biochanin A (Shajib, 2012) and daidzein (Tamura et al., 1969) can inhibit plant growth, while genistein can inhibit root absorption of nutrients (Stenlid, 1961); the allelochemical effects of the other compounds have been poorly investigated. Since biochanin A, calycosin, and prunetin persisted in sterile soil treatments at relatively high concentrations over the course of the experiment (Figure 2.6), it may be that the ~25% suppression of seed germination found in sterilized straw residue treatments at the end of our experiment (Figures 2.1 and 2.7) is due to these isoflavones. However, only the combination of live microbes and straw residues showed the prolonged, high rate of suppression that we found with fresh red clover residues (Figure 2.1), and I found low concentrations of most isoflavones in these treatments on days 16 and 30 (Figure 2.6) even though weed suppression remained relatively high. I note the relatively small microbe-by-straw interaction in the latter half of our experiment (Figure 2.2), and I propose that both chemical and microbial activity were necessary for weed suppression by straw residues. Future research that focuses on microbial interactions with bound chemicals in solid residues may lead to practices that can prolong cover crop suppression of weeds beyond the residence time of water-soluble allelochemicals in soils.

Conclusions

Even without the addition of cover crop residues, I found that the soil microbial community consistently suppressed seed germination (Figure 2.1) and seedling growth rates (Figure A.1) throughout the entire experiment. The dynamics of this non-additive microbe and residue combination call attention to the nature of specific microbial interactions with cover crop residues and their associated allelochemicals, and understanding these interactions may lead to improved biocontrol of weeds from these two sources. I found a negative interaction between activity microbial and cover crop-derived weed suppression, but the nature of this interaction was dynamic in time. As a result, overall weed suppression shifted over time from predominantly chemical phase to a predominantly microbial phase. Solid residues can prolong this initial phase, possibly by protecting water-soluble allelochemicals from microbial attack and by serving as a reservoir for water-soluble and water-insoluble compounds. The loss of residue-derived suppression over time suggests that allelochemical degradation as a major role for microbes. However, I found a consistent and high potential for microbial suppression over the course of the experiment, meaning that microbes can also play beneficial roles in weed suppression. A deeper insight into microbial community composition in cover-cropped systems may lead to a better understanding of how these beneficial roles can be stimulated to help maximize weed control.

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Figures

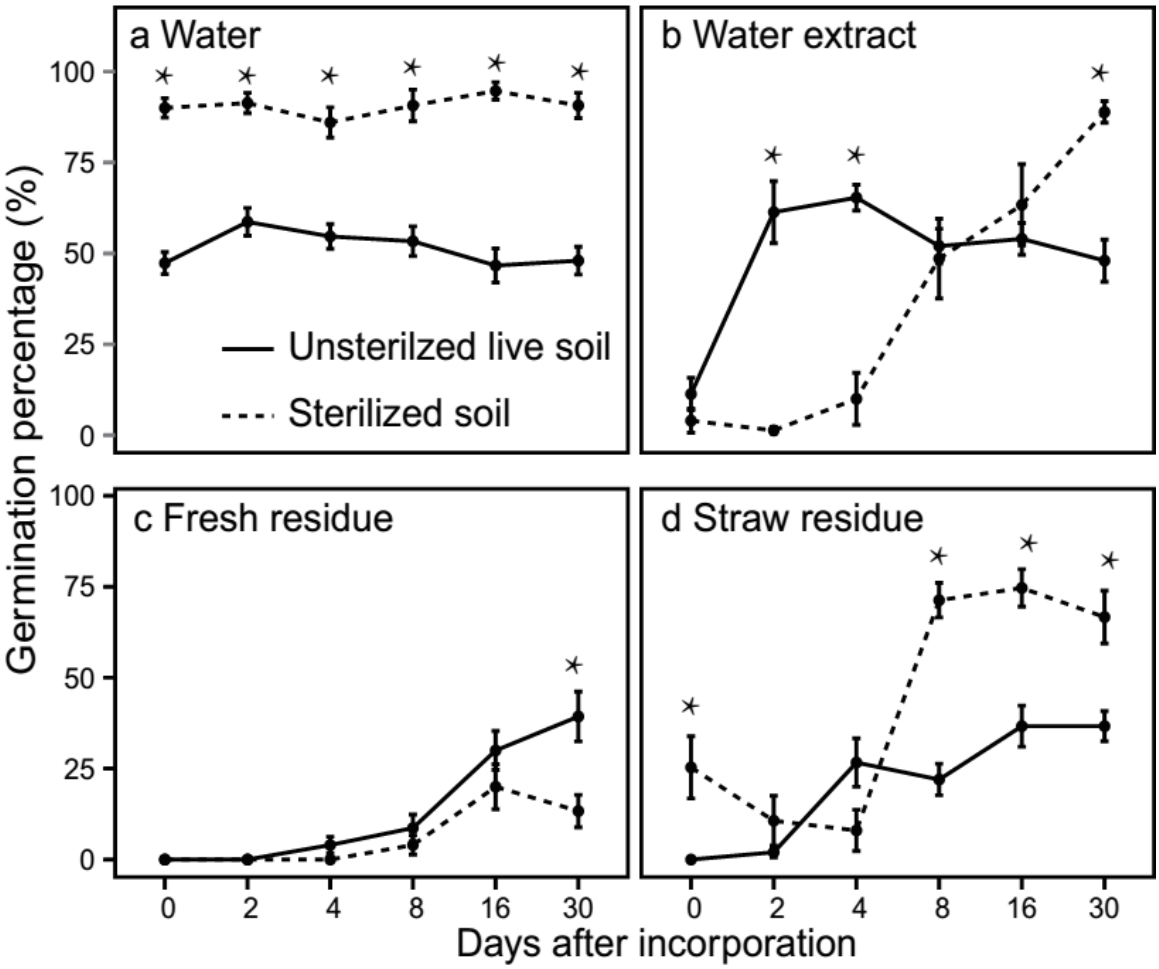

Figure 2.1. Germination inhibition by red clover residues and soil microorganisms varies over time. Percentage of germinating mustard seeds in sterilized and live soil is shown for treatments exposed to (a) water, (b) water-soluble extracts, (c) fresh residues, and (d) straw residues. Error bars are standard errors from ten replicate analyses. Stars indicate comparisons that were determined to be significantly different at alpha = 0.05 by a Tukey's HSD test.

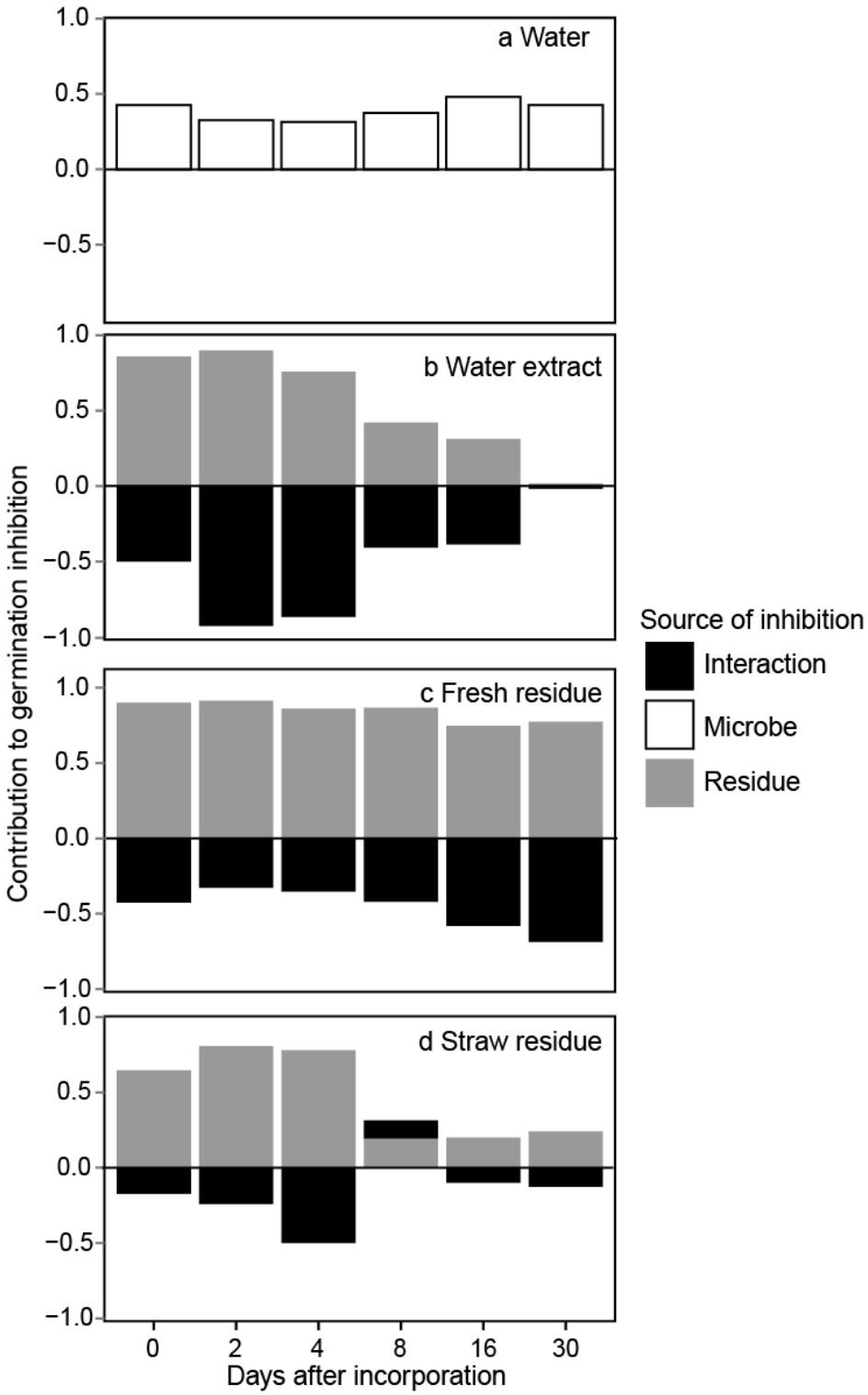

Figure. 2.2 Antagonistic interactions between soil microorganisms and red clover residues differ between residue fractions. Bars indicate the strength of microbe-only, residue-only, and microbe-by-residue contributions to germination inhibition.

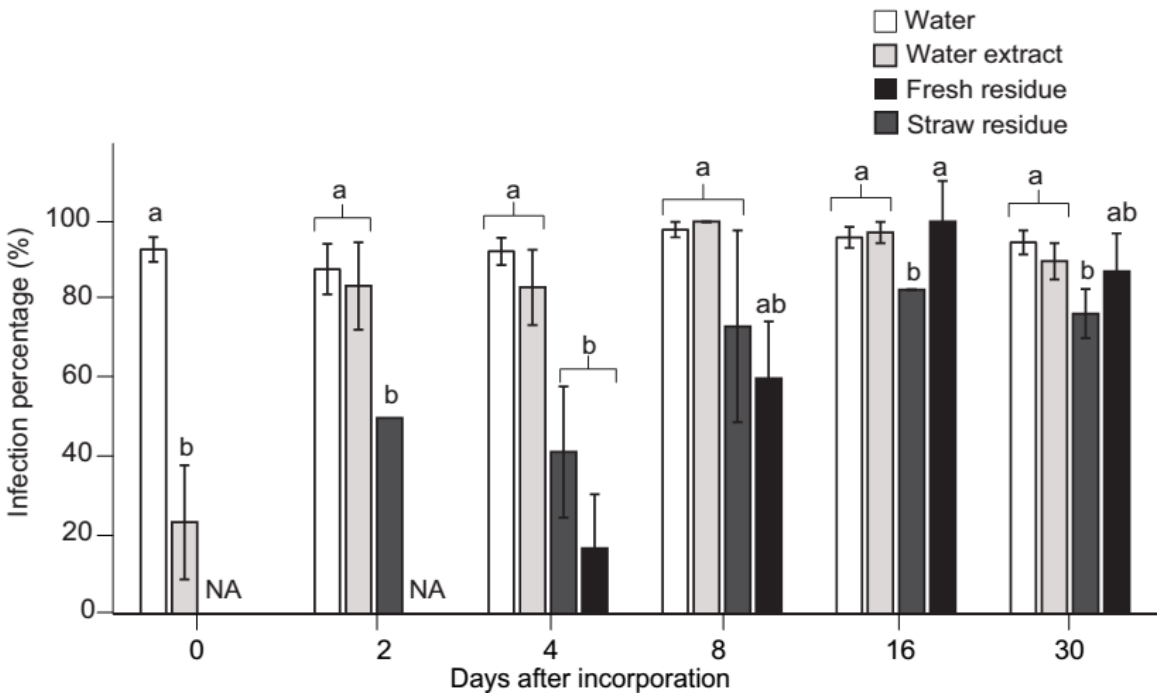

Figure. 2.3. Red clover residues inhibit seedling infection. Seedling infection percentage of mustard in live soil is shown for treatments exposed to water, water-soluble extracts, fresh residues, and straw residues. Infection percentage was measured by the number of infected seedling divided by the total number of germinated seedlings in one bioassay. NA: indicates that no infection data was available because no seeds germinated at these time points. Error bars are standard errors from ten replicate analyses. Different lowercase letters were determined to be significantly different at alpha = 0.05 by a Tukey's HSD test.

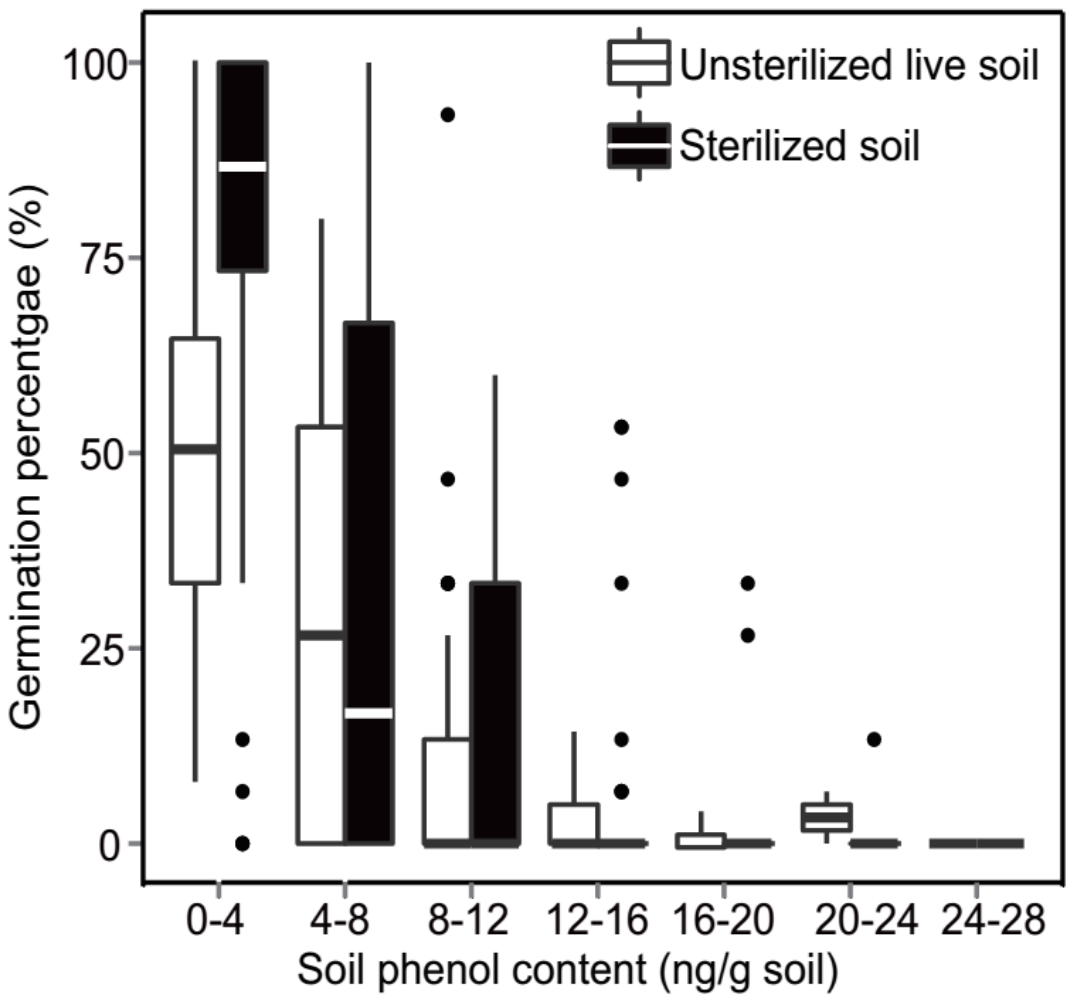

Figure. 2.4. Germination is inhibited by high concentrations of soil phenols.

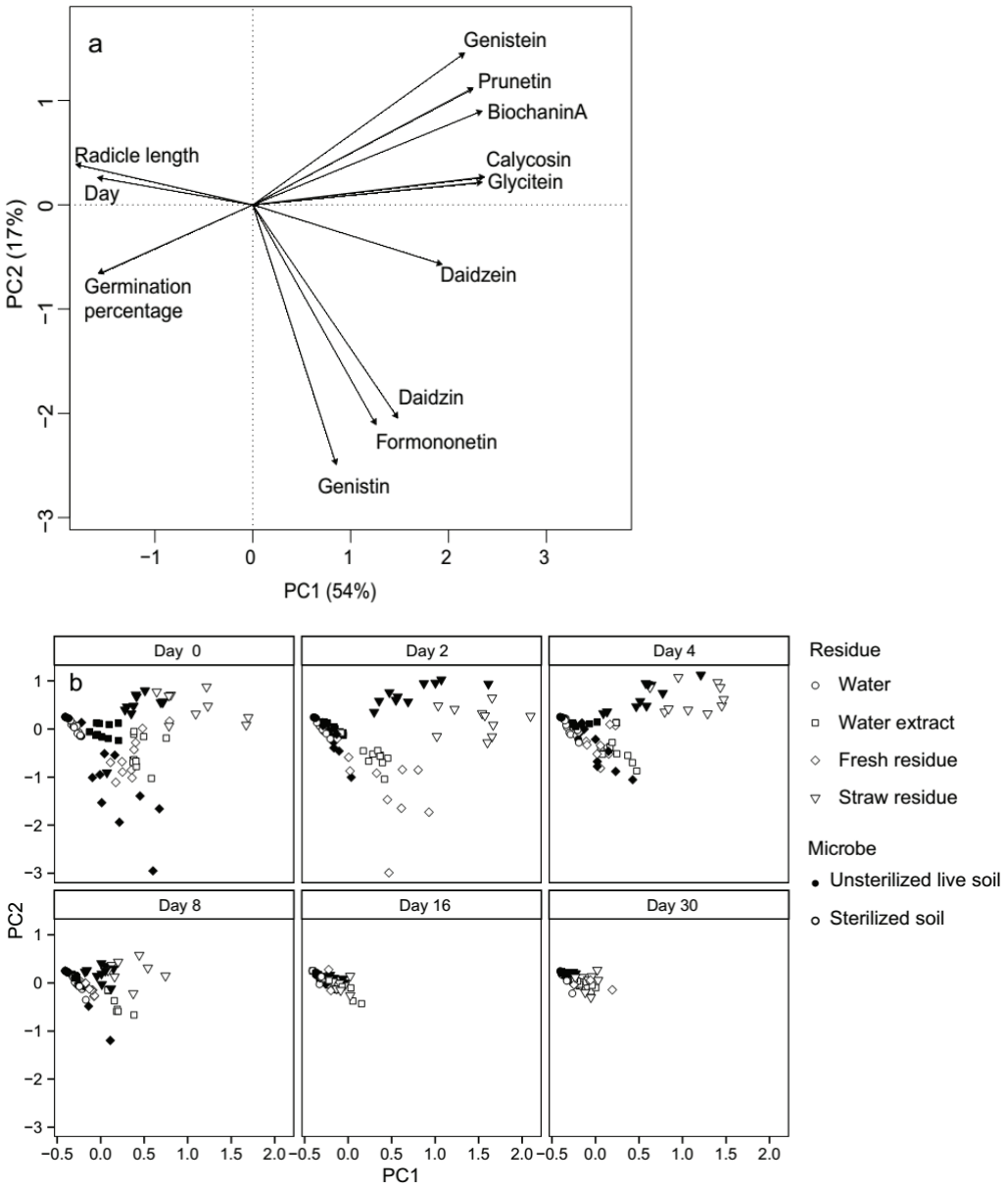

Figure. 2.5. Soil chemistry differs between residue fraction treatments. All panels were derived from a single Principal Components Analysis of HPLC-derived isoflavone concentrations in soil. Panel (a) shows the loadings of various isoflavone compounds on the ordination axis, as well as the loadings of the two main response variables (seed germination and radicle elongation). Differences in overall chemical profile between treatments is shown for different days post incorporation in panel (b), which indicates that soil chemistry of all treatments resembled that of water-only controls by the end of the experiment.

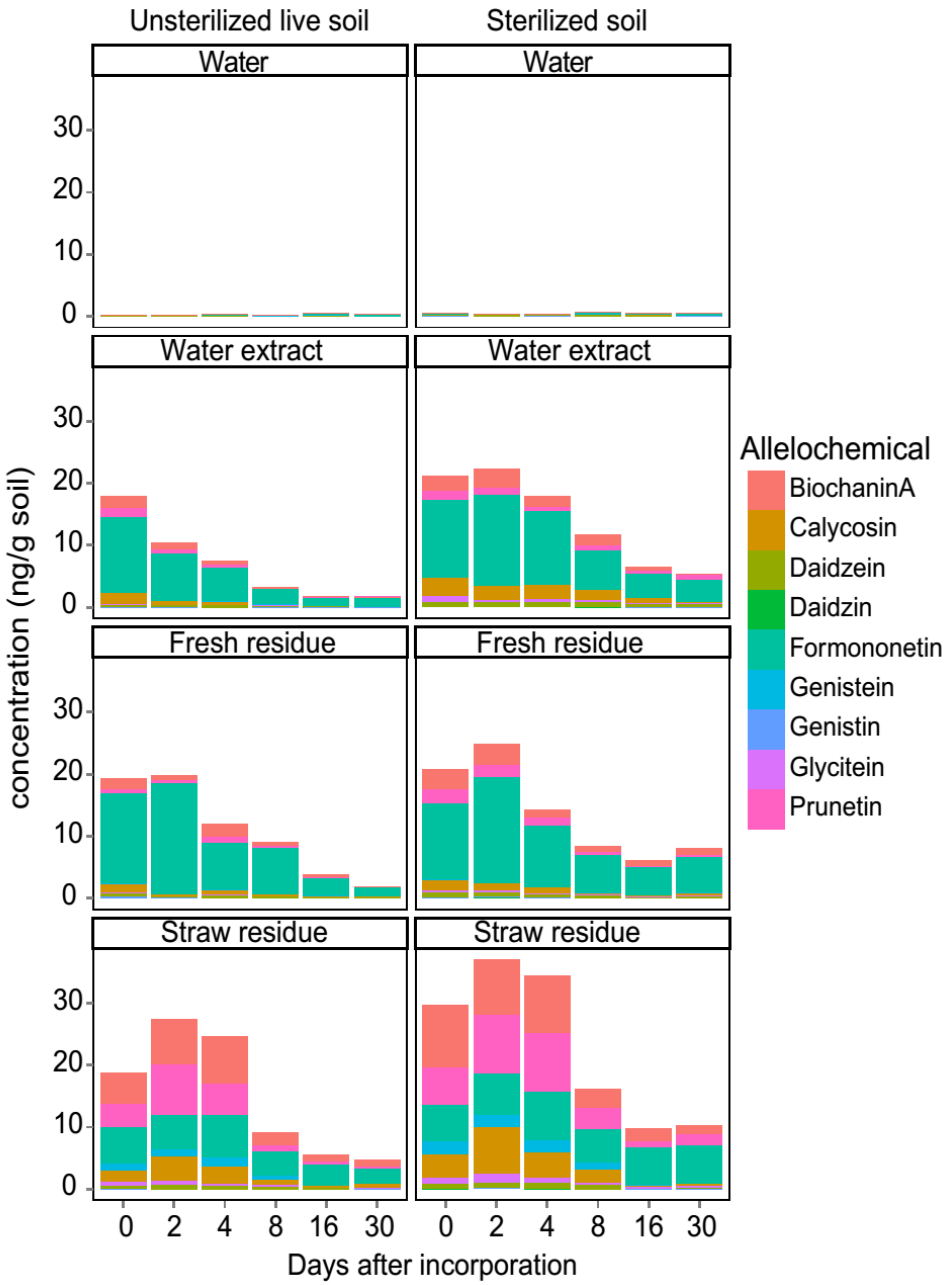

Figure. 2.6. Isoflavone content differed between treatments and over time. Stacked bars indicate the relative concentrations of nine major isoflavone components in sterilized and live soils for treatments exposed to water, water-soluble extracts, fresh residues, and straw residues.

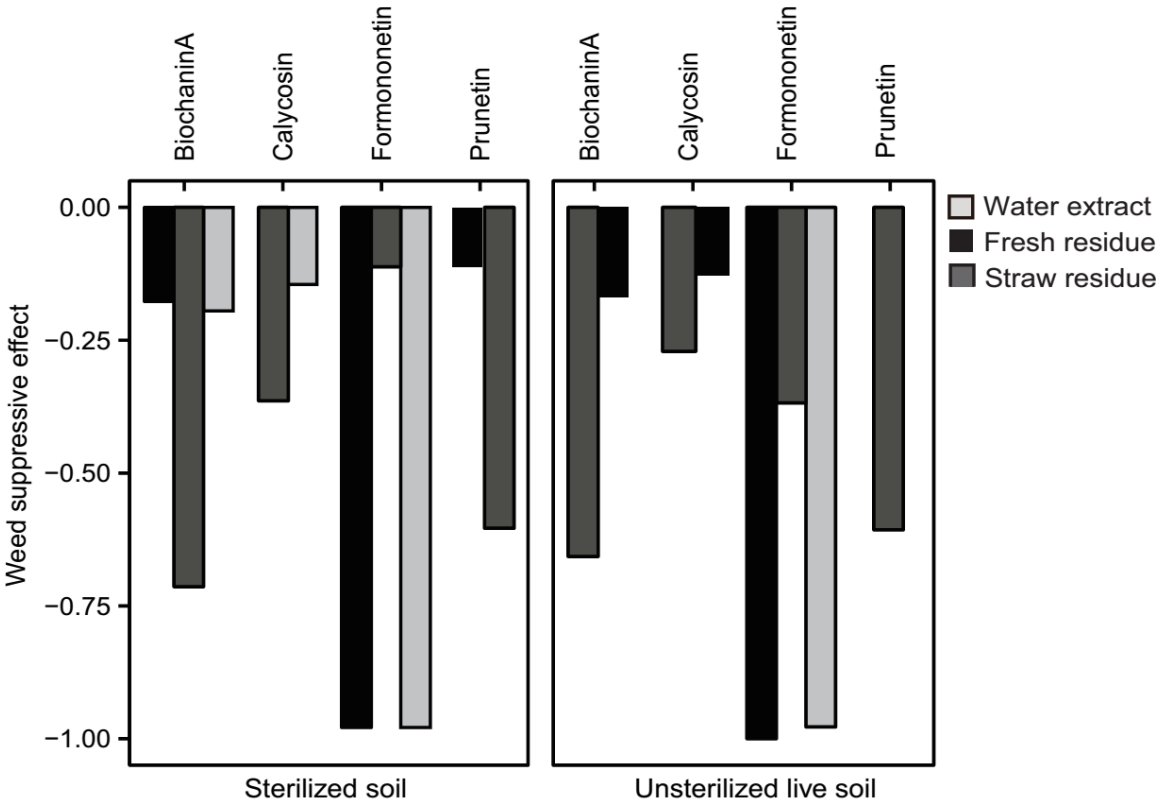

Figure. 2.7. Different isoflavones were associated with weed suppression in different treatments. The height of bars show the loadings of various isoflavone compounds on a PLSR axis describing the relationship between isoflavone concentration and seed germination rate. The negative loadings indicate that all of these isoflavones inhibited seed germination.

CHAPTER 3: USING INTERACTIONS BETWEEN COVER CROPS AND SOIL MICROORGANISMS TO IDENTIFY POTENTIAL BIOCONTROL AGENTS AGAINST WEEDS

Abstract

Biological control of weeds may help to reduce the use of herbicides and may contribute a sustainable weed management. The diversity of soil microorganisms (Roesch et al., 2007) represents an untapped resource of potential biocontrol agents for weeds. In order to take advantage of these microbial agents, it is critical to know more about their identities and activities, and about their potential for synergistic interactions with other weed control techniques like green manures. I propose that the useful weed control microorganisms will possess one or more of the following desirable traits: 1) they inhibit weed growth or promote disease incidence on seedlings; 2) they can be enriched by the addition of green manures; and 3) they are keystone players in the soil microbial community. I conducted a weed germination experiment to test how weeds and microbes respond to the addition of different soluble and insoluble fractions of green manure. I used high-throughput DNA sequencing to characterize soil bacterial and fungal communities. I conducted Partial Least Square Regression analyses to identify microorganisms that were associated with stunted and diseased seedlings. Different microorganisms participated in weed growth inhibition and disease promotion (Permutational MANOVA, $P<0.001$, for both bacteria and fungi). These weed-suppressive microorganisms were also different in different fractions of green manure residues (Permutational MANOVA, $P<0.001$, for both bacteria and fungi). The green manure addition stimulated seedling attack by bacteria mostly in the classes of *Alphaproteobacteria* and *Sphingobacteriales* and fungi mostly in the classes of *Sordariomycetes* and *Agaricomycetes*. I explored microbial co-occurrence network to identify keystone species. However, no microorganism behaved as keystone species. Instead, all microorganisms were highly connected within clusters. For the putative weed suppressive microbes identified in this study, some of them are known plant antagonists, such as *Nectriaceae* and *Pseudomonas*; however, some of them could be novel microorganisms warranting further study.

Introduction

The vast diversity of soil microorganisms (Roesch et al., 2007) represents an unexploited resource of potential biocontrol agents for agricultural weeds (Charudattan, 2001; Chee-Sanford et al., 2006). Enlisting these agents would benefit from a deeper understanding of how soil microbial communities respond to agricultural management, for example, the incorporation of cover crop residues as green manures (Bossio et al., 1998; Tamura et al., 1969). The use of green manures can stimulate microbial activities (Gunapala et al., 1998) and suppress weed emergence (Creamer et al., 1996; Liebman et al., 2000). The addition of residues from red clover, rye, oats, crimson clover and hairy vetch can stimulate some plant pathogen populations (Grunwald et al., 2000; Reeleder et al., 2006; Rothrock et al., 1995) and increase disease incidence on seedlings (Conklin et al., 2002; Manici et al., 2004). To effectively utilize the soil microbial community for weed control, it is important to know about the identities and activities of potential native biocontrol agents and to understand their interactions in weed suppressive functions and their responses to cover crop residue incorporation.

Microbial activity has been long implicated as a factor in weed suppression (Charudattan, 2001; Kremer et al., 2006; Kremer et al., 1996). In Chapter 2, I documented that the microbial community from soils under red clover cultivation could inhibit weed seed germination, reduce seedling growth rates, and promote disease incidence on seedlings. To date, much previous work that has attempted to identify important microbial biocontrol agents has primarily focused on relatively few culturable microbial groups that are known to interact with plants: mycorrhizal fungi (Jordan et al., 2000; Rinaudo et al., 2010), rhizobacteria (Kremer et al., 1990; Kremer et al., 1996) and plant pathogens (Hoagland, 2001; Mohler et al., 2012). However, given the extreme diversity of uncultivated soil microorganisms (Torsvik et al., 2002), it is likely that unknown soil microbes can participate in weed suppression through other direct and indirect pathways: competition for nutrients (Kaye et al., 1997; Kumar et al., 2008), release of inhibitory compounds (Kremer et al., 2001; Sarwar et al., 1995), and interactions with other beneficial or detrimental soil microorganisms (Matthews et al., 2001; Xavier et al., 2003). The importance of these microorganisms to weed suppression has not been discovered yet.

Modern, high-throughput DNA sequencing provides an unprecedented level of detail about whole microbial communities without the need for pure culture. However, there is no reason to assume that every microorganism in the soil community is participating in weed suppression in a significant way. Thus, along with whole-community characterization of soil microorganisms, I need to distinguish the non-actors from the potential biocontrol agents with putative weed suppressive activities. Although correlation does not prove causation, I propose that microbial taxa with a high abundance on diseased or stunted seedlings are good, first-pass candidates for putative weed suppressors.

A second useful trait of putative biocontrol agents relates to their responsiveness to soil management, for instance, by green manuring. The composition and availability of substrates within green manures change during decomposition, potentially selecting for different portions of the soil community. In general, the initial phase of decomposition consists of easily released, water-soluble chemicals and subsequent release of recalcitrant, insoluble substrates (Bonanomi et al., 2006). These soluble and insoluble fractions of residues have different impacts on microbial communities (Baumann et al., 2009; Bending et al., 2002) and their weed suppressive activities (Chapter 2). Useful native biocontrol agents should respond positively to some or all portions of cover crop residues, allowing farmers to "manage" them during some phase of cover crop decomposition.

Microbial weed suppression activities can involve complex ecological processes (Jilani et al., 2008; Nehl et al., 1997) that require microbes to interact and cooperate. Keystone species in a community play central roles in connecting other species and maintaining ecological function (Barberan et al., 2012; Faust et al., 2012). Putative biocontrol agents that are also keystone species would be particularly useful targets for manipulation, as they would help coordinate the activities of multiple weed-suppressive taxa. While interactions between uncultured microbial taxa are not well characterized, network analysis can help pinpoint microorganisms that are "hubs" of co-occurrence as potential keystone players in microbial communities (Faust et al., 2012; Steele et al., 2011). Thus, a third desirable trait for native biocontrol agents is their position as hubs in co-occurrence networks.

In this study, I aim to identify putative weed control microorganisms in uncultured soil communities based on the following desirable traits: 1) they are positively correlated with diseased or stunted weed seedlings; 2) they can be enriched by the addition of green manures; and 3) they arise as co-occurrence hubs in the soil microbial network. To discover these microorganisms, I examined the dynamics of weed seedling- and rhizosphere-associated soil bacterial and fungal communities in seedling growth bioassays subjected to amendment by different fractions of red clover residues. It is not my intention to test any specific hypotheses about microbial weed suppression, but the conceptual criteria developed in this study should provide valuable information on novel microorganisms or populations that have the potential for weed suppression in cover cropped agroecosystems.

Methods and materials

Germination bioassay

I used samples derived from my experiment that I previously detailed in Chapter 2. Briefly, I collected soil (Catlin silt loam (Oxyaquic Argiudoll) with the following characteristics: 7% sand, 68% silt, 25% clay, 4.2% soil organic carbon, pH 7.2) and the aerial portions of "Mammoth" red clover (*Trifolium pratense*, L.) from a plot in Urbana, Illinois. I processed the red clover into three different residue fractions: 1) the water-soluble fraction, 2) the insoluble "straw" fraction after water extraction, and 3) the whole, fresh residues (i.e. unprocessed plant material composed of both water-soluble and straw fractions). I passed the soil through a 2-mm sieve, and then I combined the soil with one of the three residue fractions or with sterile water (control). I used these soil + residue combinations to create seed germination bioassays (Dabney et al., 1996) using 15 seeds of a high-germinating IdaGold mustard variety (*Sinapis alba* L.) as the target species. After a 7-day incubation, I scored germinated seedlings to determine the mean seedling length for each bioassay and the number of seedlings showing necrotic tissue or other signs of infection in each bioassay. Altogether, I scored 480 bioassays, but I only consider half of these here because the other half were constructed with sterilized soil.

Seedling and rhizosphere soil microbial DNA extraction

To obtain microbial communities associated with weed seedlings, I collected all seedlings with visible necrotic tissue or other signs of infection after the bioassay incubation period. All infected seedlings from the same bioassay were pooled together for a single composite sample for each bioassay. I gently shook the seedlings to remove loosely attached soil and then collected soil still adjacent to seedlings as rhizosphere soil. I separately collected the seedlings after removing rhizosphere soil to determine which microbes were colonizing the seedlings. The whole dataset consisted of 176 rhizosphere soil samples and 176 seedling samples; this is less than the total number of bioassays reported above because some highly suppressive bioassays yielded no seedlings (0% germination rate). Soil DNA extractions used the FastDNA SPIN kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's protocol, and DNA from microbes on seedlings was extracted with the FastDNA SPIN kit (MP Biomedicals, Solon, OH) using the modified manufacturer's protocol with the CLS-Y buffer. All DNA extractions were followed by an additional purification with 1% cetyltrimethylammonium bromide and a chloroform:isoamyl alcohol extraction to remove residual soil impurities (e.g. humic acids).

Illumina sequencing

I accessed the bacterial communities by sequencing the V3 – V4 region of 16S rDNA using the PCR primers 515F and 926R (J. G. Caporaso et al., 2011; Lane, 1991). I accessed the fungal communities by sequencing the ITS2 region of ITS using the PCR primers ITS3 and ITS4 (White et al., 1990). I modified the primers with adapter sequences required by the Illumina Sequencing approach and a unique dual-index barcode was assigned to each sample. 50ul PCR reaction contained: 25uL 2X KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Woburn, MA, USA), 1uL 250 uM forward and reverse primer, 50 ng template DNA, and 21uL DNA-free water. Thermal cycling conditions for this reaction included an initial denaturation at 98 ℃ for 45 sec., 25 cycles of 98 ℃ for 15 sec., 65 ℃ for 30 sec., 72 ℃ for 30 sec., followed by a final extension at 72 ℃ for 2 min. Amplicons were purified by 0.8 X volume of AMPure® XP beads (Agencourt Bioscience, Beverly, MA, USA) and quantified by Quant-iTTM dsDNA HS Assay (Invitrogen, Carlsbad, CA, USA). Amplicons from different samples were pooled

in equimolar concentrations. The amplicon pools were sequenced by Roy J. Carver Biotechnology Center (Urbana, IL, USA) using Illumina MiSeq V3 platform instrument with a 2 x 250 bp reads configuration and Nano Kit v2 (Illumina, San Diego, CA, USA).

I merged the paired ends of raw sequence reads using the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). The merged sequences were quality filtered by requiring that 95% percent of the bases had quality scores larger than 30. I then used USEARCH version 8.0.1517 (Edgar, 2010) to do following processes: remove singletons; cluster sequences into operational taxonomic units (OTUs) based on a 97% similarity threshold; select a representative sequence for each OTU; and detect chimeric sequences using the Gold database (Kyrpides, 1999) as the reference for bacteria and the UNITE ITS database for fungi (Abarenkov et al., 2010). The representative sequences of bacteria were aligned and assigned taxonomic information by Greengenes database using *QIIME* (J Gregory Caporaso et al., 2010). Because the fungal ITS2 sequences were too variable to be aligned, the taxonomic information of fungal sequences were directly assigned by *QIIME* using the UNITE ITS database (Abarenkov et al., 2010).

Trait-based discovery of putative biocontrol OTUs

I used the following numerical tools to score microbial OTUs on three desirable traits for putative weed biocontrol agents: 1) correlation with diseased or stunted weeds, 2) stimulation by green manuring, and 3) potential keystone status. Microbial OTUs possessing all three traits were deemed to have good potential as putative weed control microorganisms. For all data analyses, the raw OTU data were subject to the Hellinger transformation (Legendre et al., 2001).

I used Partial Least Squares Regression (Carrascal et al., 2009) to find OTUs that were correlated with two dimensions of weed suppression: disease promotion and inhibition of seedling growth from the entire dataset. Partial Least Squares Regression (PLSR) is a modeling technique that can be used to relate a set of multivariate explanatory variables (microbial community composition, in this case) to a set of univariate or multivariate response variables. For our response variables, I used mean seedling length from bioassays as a metric of seedling growth, and I used the number of seedlings in a single bioassay with necrotic tissue or signs of infection as a metric of disease promotion (Chapter 2). Because inhibiting seedling growth may involve microbes

on seedlings and in rhizoshphere soil (Johnson et al., 1972), I used both microbial communities to identify seedling growth inhibiting microbes. Microbes must colonize seedling to cause disease, therefore I used microbial communities on seedlings to identify disease promoting microbes (i.e. no rhizosphere soil samples). PLSR constructs a set of orthogonal "latent variables" to maximize the covariance between the community data and the response variable. Thus, the first latent variable in the PLSR expressed microbial community turnover that was most associated with variation in seedling length or disease promotion. I selected OTUs with the top 5 percent of negative loadings on the mean seedling length latent variable as putative growth-inhibiting OTUs, and I used the loadings of OTUs along the first PLSR latent variable as the index of microbial association with one role. I selected OTUs with the top 5 percent of positive loadings on the disease promotion latent variable as putative disease-promoting OTUs. PLSR was performed in R using function plsr() in the package "pls" (Mevik et al., 2001). I used permuatational multivariat ANOVA to determine whether the putative growth-inhibiting community was different from the disease-promoting community. I classified OTUs into phylum and class. I used pairwise t-test to determine pairwise differences in level of class and phylum between the two weed suppressive roles.

To determine if putative weed suppressive OTUs differed in their response to different fractions of red clover residues, I performed the above PLSR analyses separately for each residue fraction and water. I am particularly interested in OTUs that had unique responses to one residue fraction and to water. I used Permutational MANOVA to determine whether putative growth-inhibiting OTUs were different from disease-promoting OTUs, and whether OTUs were different between residue fractions within each weed suppressive role.

In particular, I was interested in OTUs that were stimulated by at least one residue fraction, as this is a trait that indicates the potential to manage native populations of these OTUs using green manuring. I used a t-test to compare the relative abundance of each putative growth-inhibiting or disease-promoting OTU in the water control treatment vs. its relative abundance in a particular residue addition treatment. I calculated P-values of the difference with two-sided Fischer's exact test and corrected it with Benjamini–Hochberg's false discovery rate (Benjamini et al., 1995).

I used network analyses to examine the co-occurrence pattern of putative growth-inhibiting and disease-promoting microbes. I considered a valid co-occurrence correlation between OTUs if the Spearman's correlation coefficient (r) was both > 0.7 and statistically significant (P-value <0.01) (Barberan et al., 2012). I analyzed the number of nodes, connectivity, clustering coefficient, and modularity of the microbial community network to infer the size of the network, interactions between the microbial OTUs and possible ecological niches. I used leave one out method to evaluate "degree of keystoness" of a OTU (Berry et al., 2014). I set the abundance of one OTUs to zero and re-constructed the network. I compared the number of OTUs in the new network with the original network to evaluate the impact of the OTUs on species richness, which is the number of OTUs that are no longer connected to the new network lacking the left-out OTU. I calculated the topological features for each OTUs. I calculated four topological feature (degree, betweenness centrality, closeness centrality and transitivity) for each OTUs to describe its characteristics in the network. I considered OTUs to be potential keystone taxa if they had: high impact on species richness, high mean degree, low betweenness centrality, high closeness centrality, and high transitivity (Berry et al., 2014). Statistical analyses were carried out in the R in package "igraphy" (Bastian et al., 2009).

Results

Putative weed suppressive microbes

Looking across all treatments and all samples, I found 286 bacterial OTUs and 115 fungal OTUs with high correlations to diseased incidence or poor seedling growth. Disease promotion and growth inhibition roles were played by different putative microbial OTUs (Permutational MANOVA, P-value <0.001, for both bacteria and fungi). Only 30% of bacterial OTUs and 25% of fungal OTUs shared both roles. At the phylum level, these two roles were both dominated by phylum of *Proteobacteria, Bacteroidetes and Firmicutes* (Figure 3.1a). The most abundant phyla, *Proteobacteria,* represented 65% of the disease-promoting community and 60% of the seedling growth inhibiting-community. The relative abundance of major fungal classes varied more between the two roles than did bacterial phyla (Figure 3.1b). For example, the relative abundance of

Sordariomycetes was much higher in the seedling growth-inhibiting community (52%) than in the disease-promoting community (24%) (T-test, P-value <0.001). In contrast, the relative abundance of *Mortierellales*, was lower in the seedling growth-inhibiting community (0.2%) than in the disease-promoting community (35%) (T-test, P-value <0.001).

Putative weed suppressive microbes in different residue fractions

To understand the influences of different residue fractions on the putative weed suppressive microbes, I identified the putative weed suppressive microbes in each residue fraction and water control separately. I found the residue fraction had significant impacts on the putative weed-suppressive microbial communities (Permutational MANOVA, P-value < 0.001, for both roles, and both bacteria and fungi). About one-third to more than half of microbial OTUs uniquely responded to one residue fraction (Figure B.1).

I further identified microbial OTUs that uniquely responded to one residue treatment to understand how weed-suppressive microbes interacted with different residue fractions (Figure 3.2). For the disease-promoting microbial community, all bacterial phyla were significantly different by residue fraction (ANOVA, P-value<0.01) (Figure 3.2a). For example, the bacterial OTUs that uniquely responded to the water-soluble extracts were dominated by *SPAM* (68%), which was not very abundant in the other three fractions. Like the bacterial phyla, all the fungal classes were also significantly different by residue fraction (ANOVA, P-value<0.01) (Figure 3.2b). For example, OTUs in *Incertaesedis* (67% of these were in the order *Mortierellales*) were only highly abundant in the community that uniquely responded to the fresh residues.

For growth-inhibiting microbial communities, the relative abundance of bacterial phyla between the water controls and water-soluble extracts were similar (Figure 3.2a). They were both dominated by *Actinobacteria* (about 60%) and *Bacteroidetes* (about 10%), but the OTUs within each phylum were different (Permutational MANOVA, P-value<0.01) (Figure B.1a). For the OTUs that uniquely responded to the fresh and straw fractions. *Bacteroidetes*, *Proteobacteria* and *Firmicutes* were the top 3 abundant phyla. However, the relative abundance of fungal classes varied between treatments (Figure 3.2 b). All fungal classes were significantly different by residue fraction (ANOVA, P-value<0.01). The most abundant class that was unique to water-soluble extracts was

Dothideomycetes (38%). Most of OTUs uniquely responding to fresh residues were *Sordariomycetes* (22%) and family of *Mortierellales* (33%). 48% of OTUs uniquely responding to water control were *Eurotiomycetes* (30%) and *Leotiomycetes* (18%).

In the same residue fraction, the composition of microbial communities was also different between two microbial roles (Permutational MANOVA, p-value < 0.001, for all residue fractions) (Figure 3.2). For example, for the bacterial communities unique to water-soluble extracts, the relative abundance of *Actinobacteria* was much higher in the seedling growth-inhibiting community (35%) than on the disease-promoting community (3%). On the contrary, the relative abundance of *SPAM* ranged from 2% -35% in the disease-promoting community, but no OTUs in *SPAM* were found in the seedling growth-inhibiting community.

Responses of putative weed suppressive microboes to green manure

From my initial pool of putative weed-suppressive OTUs in each residue fraction, I found the abundance of some OTUs were significantly enriched or depressed in residue treatments compared to water controls. For disease-promoting communities, 17% of fungal OTUs and 23% of bacterial OTUs changed, and for seedling inhibiting-communities, 13% of fungal OTUs and 20% of bacterial OTUs changed.

For disease-promoting microbes, overall, red clover residue addition enriched bacterial OTUs mostly in the classes *Sphingobacteriales* (19%), *Alphaproteobacteria* (17%) and *Gammaproteobacteria* (17%) and fungal OTUs mostly in the classes *Sordariomycetes* (37%) and *Agaricomycetes* (35%) and *Dothideomycetes* (10%). These top three classes were the same for seedling growth-inhibiting microbes. But the magnitudes of increase differed a lot among residue fractions (Figure 3.3). All the major bacterial classes were enriched most strongly in fresh residue treatment while *Agaricomycetes* was the only fungal class that increased most strongly by fresh residue. Water-soluble exacts enriched *Gammaproteobacteria* and *Sordariomycetes* more than other microbial groups, and straw residues enriched *Gammaproteobacteria* and *Sordariomycetes* mostly.

For bacteria, the depressed classes were similar across residue fractions and two roles. Overall, OTUs in *Flavobacteria* (41%), *Betaproteobacteria* (21%) and

Gammaproteobacteria (13%) were the top three depressed classes (Figure 3.3). The depressed fungal classes varied a lot among residue fractions and also between two roles. For example, overall the relative abundance of *Sordariomycetes* was suppressed the most strongly, but 89% of the decreased abundance was from seedling growth-inhibiting OTUs in the straw residue treatment. Similar to order *Mortierellales*, 95% of the decreased abundance was from disease-promoting OTUs in the fresh and straw residues.

Co-occurrence pattern of putative weed suppressive microbes

From the putative weed-suppressive OTUs that were common in all residue treatments, I found that 31% of disease-promoting microbes were highly correlated, and 15% of seedling growth-inhibiting microbes were highly correlated. Unlike my expectation, no microbial taxa processed the following topological features of keystone species: high impact on species richness, high mean degree, low betweenness centrality, high closeness centrality, and high transitivity. Removing an OTU disconnected no more than three other OTUs from the network. Thus, I could not identify any OTUs as keystone species in either putative growth-inhibiting or disease-promoting networks.

The topology of the two functional networks was substantially different (Table 1). The network of disease-promotion involved more OTUs with higher connections than the network of growth-inhibition. In the disease-promoting network, the top three fungal classes were *Agaricomycetes* (32%), *Eurotiomycetes* (16%) and *Dothideomycetes* (15%), and the top three bacterial classes were *Actinobacteria* (21%), *Acidobacteria* (13%) and *Alphaproteobacteria* (12%). In the seedling growth-inhibiting network, the top three fungal classes were *Sordariomycetes* (29%), *Agaricomycetes* (15%) and *Dothideomycetes* (15%). Bacterial OTUs were mostly in *Proteobacteria* (37%) *Acidobacteria* (23%) and *Planctomycetes* (20%). Bacterial OTUs from the same phylum tended to be correlated together in seedling growth-inhibiting networks. Almost all the correlations in networks (100% in growth-inhibition and 99% in disease-promotion) were positive.

Discussion

The promotion of natural borne soil communities to control weeds may provide an alternative approach to encourage biocontrol microbes without inoculation. Traditional

weed biocontrol approach is inoculation of microbial biocontrol agents into the soil (Hasan et al., 1990; Kennedy et al., 1996). However, many microorganisms present in the soil are not accessible to traditional selections of weed control agents just based on cultivation approach (Charudattan, 2001; Kennedy et al., 1996). The weed-suppressive potentials of natural soil microbial communities are largely unexplored (Chee-Sanford et al., 2006; Kremer, 1993). In this study, I harnessed the power of the sequencing approach to screen the whole microbial community for putative weed-suppressive organisms. I applied a series of selections to narrow down the super diverse whole microbial community to a list of microbe taxa that may be associated with desirable weed-suppressive traits. Here I discuss the ecology of these microbial taxa, focusing on some key players that may ultimately assist us in the selection of microbial weed control agents.

Weed suppressive microorganisms and their responses to different residue fractions

The two weed suppressive activities were performed by different microbial communities (Figure 3.1). Distinctive microbial communities observed for two activities may be influenced by the different mechanisms of disease promotion and growth inhibition. Although the length of diseased seedlings were shorter than healthy seedlings (Chapter 2), disease incidence may be just one of many forms of microbial inhibitory effects on plant growth. Microbes can inhibit root and shoot growth and yet cause no visually obvious disease symptoms (Nehl et al., 1997; Schippers et al., 1987). For example, rhizosphere bacteria can inhibit plant growth by competing with the plant for nutrients (Baas, 1990). Some growth-inhibiting bacteria, including *Pseudomonas* and *Flavobacterium,* can produce phytotoxins and phytohormones that inhibit plant growth (Nehl et al., 1997). For fungi, the relative abundance of *Sordariomycetes* was much higher in the growth-inhibiting communities than the disease-promoting communities. Because most OTUs can only be classified at the level of order, it is not clear why *Sordariomycetes* were important in delaying seedling growth but not in promoting disease.

Theoretically, the combination of soluble (water extract) and insoluble (straw) fractions of residues should be equivalent to the fresh residue. The microbes that responded to the soluble or insoluble fraction should be subsets of microbes that responded to the fresh residue. However, one-third of the microbial OTUs were unique to

each residue fraction (Figure B.1). One possible reason is that residue fractions significantly changed the whole soil microbial communities (Table B.1), which impacted the availability of putative weed-suppressive microbes. Some microbial groups, such as *Actinobacteria* and *Mortierellales*, were dramatically different between residue fractions. These microbes might be sensitive to certain types of substrates. For example, in seedling growth-inhibiting communities, more *Actinobacteria* (67% in *Actinomycetales*) were unique to water-soluble extract treatments. *Actinomycetales* has been shown to strongly prefer labile carbon over recalcitrant carbon (Goldfarb et al., 2011).

I also noticed that some microbial OTUs were common between more than one residue fraction (Figure B.1). OTUs shared between fresh residues and water-soluble extracts may be responding to the water-soluble chemicals that leak out of the fresh residues. Similarly, OTUs shared between fresh residues and straw residues, they may be associated with insoluble and more recalcitrant substrates in the fresh residue. The OTUs in both water-soluble extracts and straw residues may be generalists that can use both insoluble and soluble substrates.

The residue-inhibited microbes (Figure 3.3) supported the negative residue-microbes interactions found in my previous study (Chapter 2). These inhibitive effects on putative pathogenic microbes may be due to the anti-microbial properties of many phenolic compounds in red clover (Daayf et al., 2012; Reynolds et al., 2003). Microbes that are able to degrade phenolics may be less likely to be inhibited. For example, I discovered that only 17% OTUs in the *Nectriaceae* family were inhibited by residues, and two of the top ten enriched disease promoting OTUs were in the *Nectriaceae* family (Table 3.2). This finding may be related to the fact that pathogen species *Nectria haematococca* in this family can produce enzymes to degrade antibiotics from legumes (Daayf et al., 2012; Morrissey et al., 1999). Therefore, incorporation of cover crops rich in phenolics may select microbial communities that are resistant to phenolic antibiotics (Blum et al., 2000; Sparling et al., 1997).

Although the overall residue-microbe interactions on weed suppression were always negative through the experiment (Chapter 2), the findings of residue-stimulated putative weed-suppressive microbes are not counter-intuitive (Figure 3.3). Because the negative interactive effects on weed-suppression may partially be attributable to the

microbial degradation of allelochemicals (Inderjit, 2005; Macias et al., 2004), the stimulated microbes in residue treatments may degrade and use allelochemicals as a carbon resource. The stimulated microbial communities were different among residue fractions, which may be related to the different compositions and availabilities of substrates in different residue fractions. The abundance of the top 5 enriched bacterial classes was increased more by the fresh residues than straw and water-soluble extracts (Figure 3.3). One possible reason is that fresh residues provided the largest amount and longest lasting carbon resources, while water-soluble extracts only contained easily-degraded, soluble carbon resources. The bigger carbon resource reservoirs of fresh residue may allow more pathogens to grow. Among all the enriched OTUs, abundant classes, including *Alphaproteobacteria, Gammaproteobacteria* and *Sphingobacteria,* are the important plant tissue decomposers (Li et al., 2012). Among fungi, *Agaricomycetes* was primarily enriched in the fresh residue treatment (Figure 3.3b). This class is known for high decomposition ability (Floudas et al., 2012). I also discovered that the straw residues primarily changed OTUs in class *Sordariomycetes* (Figure 3.3b), but the OTUs belonged to very diverse genera. Thus, the responses of *Sordariomycetes* to straw residues may be very species-specific. These residue-stimulated microbes suggest that some microbes can positively respond to the addition of residues even when the overall microbe-residue interaction is negative. The composition of chemicals in residues may be a very important factor in influencing the microbe-derived weed suppression. Future investigation is warranted into the specific interaction between individual allelochemicals and microbial species.

Given the enormous diversity of microbial communities I detected here, the networks of seedling inhibition and disease promotion are small, containing relatively few phyla and classes, but also highly connected. However, based on the topological features of "keystone" species that were proposed by previous studies, I did not discover any microbial taxa that had a strong impact on the species richness of network. Instead, microbial taxa were highly connected within modules, which made the network were very resistant to loss of OTUs. These highly-connected microbes with strong module memberships may be functionally redundancy, suggesting that losing any of them won't affect the whole function of network (Barberan et al., 2012; Zhou et al., 2011).

Alternatively, it is also possible that this microbial consortium is necessary for developing plant disease. For example, *Pseudomonas* and *Enterobacteriales* are linked in the growth-inhibition network. Two non-pathogenic bacterial species belonging to *Enterobacteriales* were critical for plant diseases caused by pathogenic *Pseudomonas savastanoi* (Hosni et al., 2011). Other studies found that the mixture of multiple fungal pathogens can achieve weed suppression superior or comparable to that of individual pathogens (Chandramohan et al., 2001; Chandramohan et al., 2002). Because microbial consortia were mostly formed by fungal taxa belong to *Ascomycota* and *Basidiomycota*, and bacterial taxa belong to *Acidobacteria* and *Proteobacteria*, farmers could select multiple species in these phylogenetic groups to achieve a broad spectrum of weed control without the loss of efficacy and host specificity of an individual pathogen. However, it should be stressed that the networks are entirely based on correlation but not on function. This means, the underlying factors driving the correlations are unknown. Other factors driving positive correlations in my study include time, soil chemicals, or some interactions between microbes. Further work is needed to confirm the ideas proposed here.

Microorganisms with desirable weed suppressive traits in green manure systems

Microbial networks showed that no microbial OTUs behaved as network "hubs." Therefore, I listed the top three abundant OTUs that had the traits of weed suppression and positive responses to green manure (Table 3.2).

One of the most important attributes of the highthroughput sequencing approach is the potential to identify unknown plant antagonists. For the putative weed suppressive microbes discovered here, some of them have known pathogenicity related traits. For example, the most abundant weed-suppressive OTU belonged to the genus *Pseudomonas* (Table 3.2). The mobility of *Pseudomonas* based on chemotaxis toward exudate components is an important trait for root infection (Yao et al., 2006). Another abundant weed-suppressive OTU belonged to order *Sphingobacteriales* (Table 3.2). *Sphingobacteriales* recently has been shown to have strong chemotaxis to organic matter in marine systems (Khodadad et al., 2011). Chemotaxis is an important trait for pathogens to identify and colonize host plants (Hawes et al., 1989; Yao et al., 2006). 62% of the diseases-promoting fungi OTUs belonged to *Sordariomycetes*. The most abundant

disease promoting fungal taxa is *Myrothecium verrucaria*. It has been developed as biocontrol agent to suppressive kudzu (*Pueraria lobata*). *Myrothecium verrucaria* produces mycotoxins, which can cause the degradation of health in the kudzu vine within twelve hours of application (Boyette et al., 2002). Two abundant weed-suppressive OTUs were in the family of *Nectriaceae*. Many species (e.g. *Fusarium oxysporum, Nectria cinnabarina*) in this family are famous plant pathogens (Houston, 1994; Ploetz, 2006). However, I have little information on the interactions with plants with some putative microbes (E.g. *Dyadobacter, Geminibasidiaceae*). These microbial taxa may be special targets in future efforts to understand weed – microbe interactions.

Conclusion

The conceptual framework developed in this study proposed some candidate weed-suppressive microbes. Some of them are members of known plant antagonists, such as *Nectriaceae* and *Pseudomonas*. Some of them could be novel antagonists that are worth further study on their ecological information and actual weed-suppressive effects. I believe that the only way to provide a functional understanding of the microbiome is by cultivation and inoculation experiments. Yet, my taxonomic to phenotypic analysis via the use of sequencing does provide a starting point and hint at the potential weed-suppressive soil microbiome for future experiments.

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Tables and figures

Table 3.1. Network analysis of disease-promoting and seedling growth-inhibiting microbes.

Role	Co-occurrence Pattern (standardized c-score)	Network size [a]	Average Connectivity [b]	Average geodesic distance [c]	Average clustering coefficient [d]	Modularity [e] (no. of modules)
Disease promotion	0.20 *	336	99.3	1.96	0.86	0.13 (9)
Seedling growth inhibition	0.22 *	157	29.4	1.42	0.78	0.14 (16)

* The co-occurrence pattern is statistically non-random

a. Number of OTUs in the network

b. Average number of links of a node to other nodes

c. Average shortest path between two nodes

d. The degree of clustering

e. The strength of division of a network into modules

Table 3.2 Top abundant fungal and bacterial OTUs that fulfill weed-suppressive criteria: have weed-suppressive role and enrichment in residue treatments.

OTU	Domain	Phylum	Class	Order	Family	Genus	Species	Residue	Role
X3	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae			Fresh	Disease promotion
X18	Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium		Fresh	Disease promotion
X394	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		Fresh	Disease promotion
X3	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae			Fresh	Seedling growth inhibition
X5	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium		Fresh	Seedling growth inhibition
X18	Bacteria	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Sphingobacteriaceae	Sphingobacterium		Fresh	Seedling growth inhibition
X4	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Incertaesedis	Myrothecium	Myrotheciu m verrucaria	Straw	Disease promotion
X5	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae			Extract	Disease promotion
X18	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae			Fresh	Disease promotion
X10	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae	Rhizoctonia		Fresh	Seedling growth inhibition
X2	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium		Straw	Seedling growth inhibition
X18	Fungi	Basidiomycota	Agaricomycetes	Cantharellales	Ceratobasidiaceae			Fresh	Seedling growth inhibition

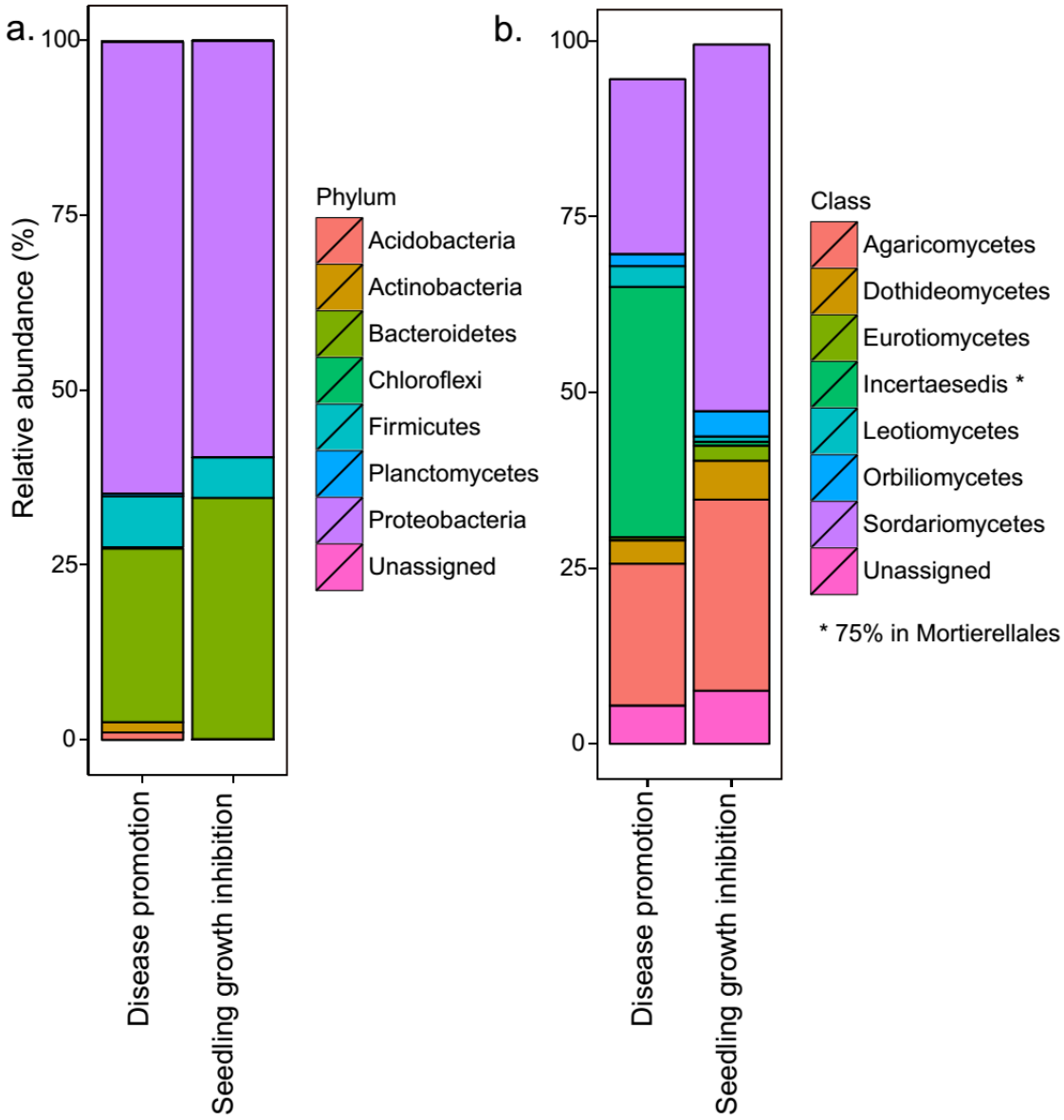

Figure 3.1. Relative abundance of major (a) bacterial phyla and (b) fungal classes with putative weed-suppressive roles. Sum of relative abundance was standardized into 100%.

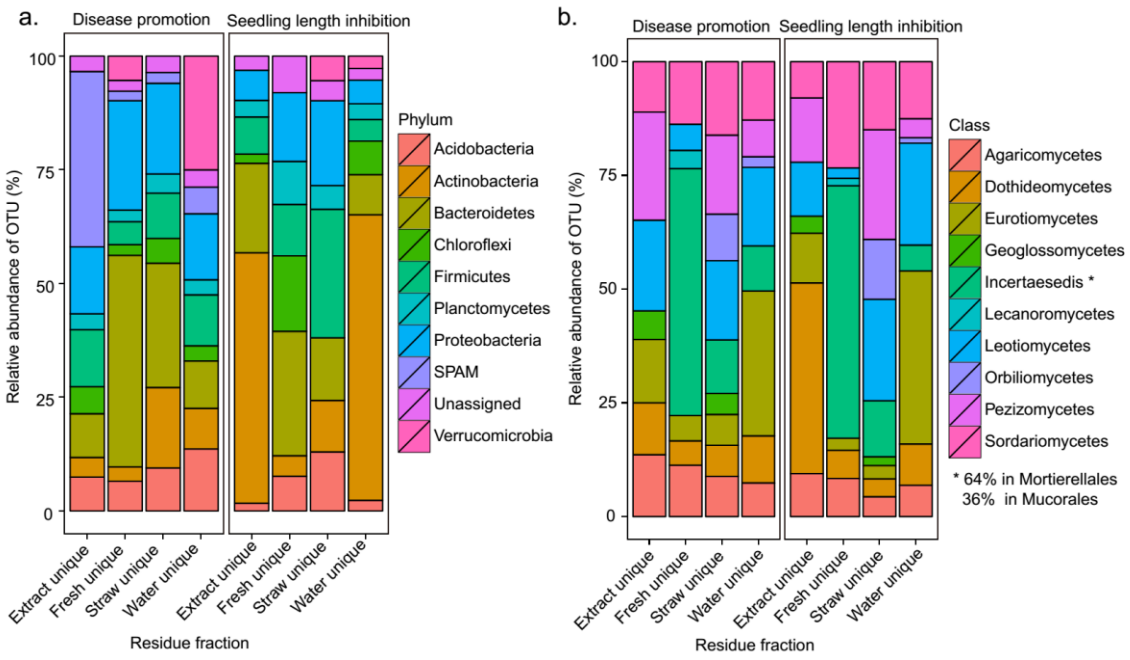

Figure 3.2. Relative abundance of major (a) bacterial phyla and (b) fungal classes with putative weed-suppressive roles that were unique to different residue fractions or water control. Sum of relative abundance was standardized into 100%.

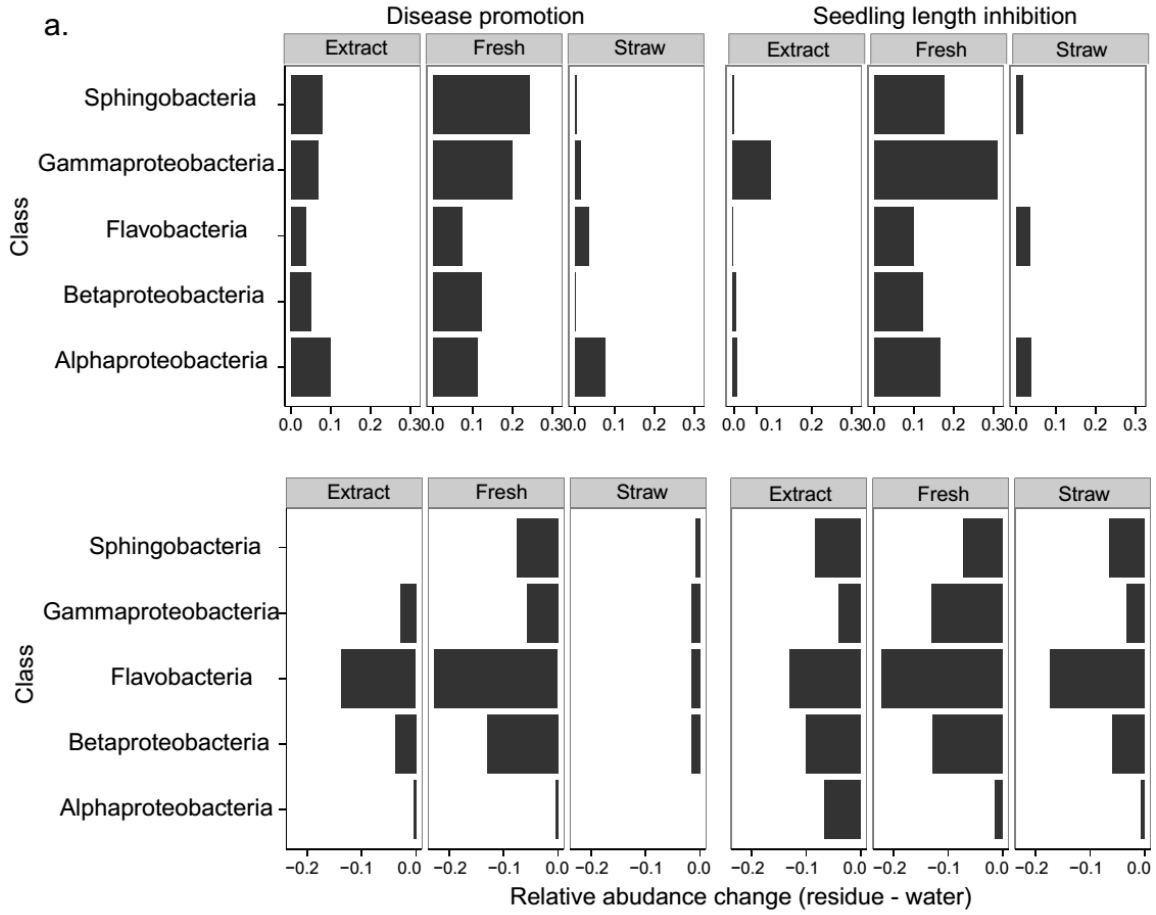

Figure 3.3. Stimulation (top panels) and suppression (bottom panels) of major (a) bacterial and (b) fungal classes with putative weed-suppressive roles. Stimulation and suppression are based on the difference in relative (Hellinger-transformed) abundance of OTUs between residue fraction treatments and sterile water controls. Only OTUs with statistically non-zero abundance changes (based on $p < 0.05$ with Benjamini-Hochberg false discovery rate) are included. Horizontal bars show the sum of all positive or negative abundance changes for each group. For example, water-soluble extract treatments were more suppressive than stimulatory for putative disease-promoting Sphingobacteria.

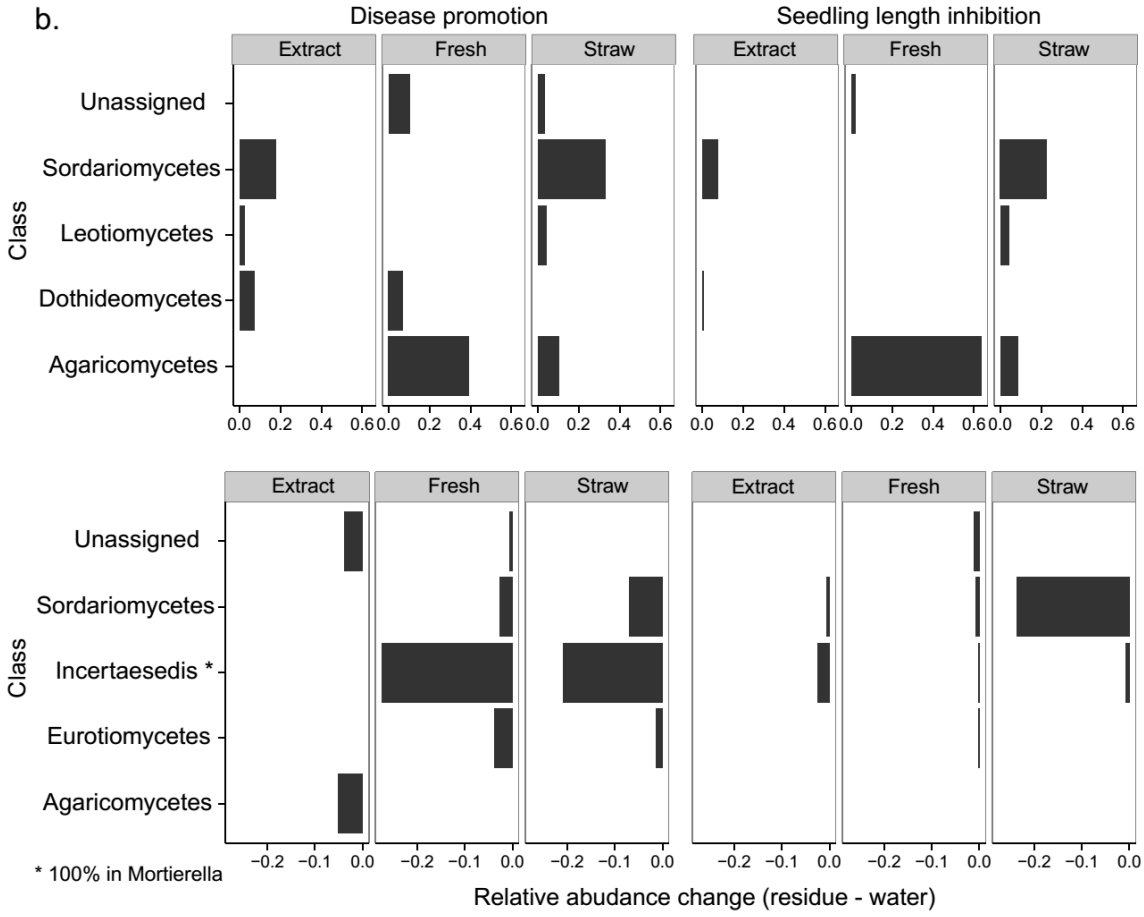

Figure 3.3. Stimulation (top panels) and suppression (bottom panels) of major (a) bacterial and (b) fungal classes with putative weed-suppressive roles. Stimulation and suppression are based on the difference in relative (Hellinger-transformed) abundance of OTUs between residue fraction treatments and sterile water controls. Only OTUs with statistically non-zero abundance changes (based on $p < 0.05$ with Benjamini-Hochberg false discovery rate) are included. Horizontal bars show the sum of all positive or negative abundance changes for each group. For example, water-soluble extract treatments were more suppressive than stimulatory for putative disease-promoting Sphingobacteria.

CHAPTER 4: ALLELOCHEMICAL AND MICROBIAL WEED SUPPRESSION UNDER DIFFERENT AGRICULTURAL MANAGEMENT SYSTEMS

Abstract

Management of agricultural systems is known to change soil microbial communities. Consequently, these changes may lead to different levels of weed suppression from the soil-borne microorganisms and cover crop residues. The objectives of this study were to evaluate the effects of three agricultural management systems (tillage, no-tillage and organic systems) on residue-derived and microbial weed suppression, and to identify putative weed-suppressive microbes and their responses to the addition of fresh residues in each system. Overall, organic and tillage systems offered higher cover crop- and microorganism-derived weed suppression than the no-tillage systems. Different microbial communities were associated with seed germination suppression and seedling growth suppression. For these putative weed-suppressive microbes, more microbes showed negative responses to the addition of cover crop residues. However, the microbes in organic soil had the smallest negative responses and more connections among individual taxa than the microbes in tillage and no-tillage systems. Some of the putative weed-suppressive microbes are members of known pathogens, such as *Fusarium*, *Rhizoctonia solani*, *Enterobacte.* Some of them may be novel pathogens that have not been known yet. These results suggest a potential to optimize weed suppression by managing soil microbial communities. The putative weed-suppressive microbes identified here provide a basis for promoting biocontrol agents in cover crop agricultural systems.

Introduction

Soil microorganisms have been suggested to be important in weed management in agricultural systems (Charudattan, 2001; Chee-Sanford et al., 2006; Kennedy et al., 1996). Soil microorganisms can suppress weeds directly by infection or indirectly by exuding toxic compounds (Charudattan, 2001; Kennedy et al., 1996). Soil microorganisms also mediate the activities of allelochemicals from cover crop residues (Inderjit, 2005; Kaur et al., 2009), which are important in weed suppression efforts (Liebman et al., 2000; Macias et al., 2007; Singh et al., 2003). Agricultural management systems strongly impact the soil microbial community composition (Drijber et al., 2000; Kandeler et al., 1999), and these changes may, in turn, influence the interaction effects of residue and microbes on weed suppression in systems under different management regimes. In order to utilize the fresh residue-derived allelochemicals and microbes for weed biocontrol, it is important to understand how these weed suppression potentials change across a range of management regimes.

Soil microbial communities respond to crop and soil management practices such as tillage (Feng et al., 2003; Kandeler et al., 1999) and residue incorporation (Carrera et al., 2007). Generally, no tillage reduces the physical disturbance of the soil, enhances organic matter accumulation, and conserves moisture, which often leads to an increase in microbial biomass in comparison to tilled systems (Doran, 1980; Kandeler et al., 1999; Six et al., 2006). Organic farming substantially reduces the use of synthetic fertilizers and pesticides. These practices have positive impacts on soil microbial communities, such as higher functional diversity and resource utilization efficiency (Mader et al., 2002; Moeskops et al., 2010).

The changes in microbial communities are widely observed with changes in agricultural management systems (Drijber et al., 2000; Feng et al., 2003; Spedding et al., 2004), and weed-suppressive microbes may be altered with the management system as well. But the directions of changes in suppression is inconsistent in the literature. Increased frequency of plant disease was observed in some conservation tillage (Cook et al., 1991; Kremer et al., 1990) and organic farming fields (Ngouajio et al., 2003). These fields leave plant debris on the top layer of soil, which may promote the survival of deleterious rhizobacteria. In contrast, other studies found that plant diseases were reduced

in reduced tillage compared to conventional tillage (Janvier et al., 2007; Tinline et al., 1991). These authors speculated that these practices increase microbial populations sizes, creating a resource-limited environment with possible competition for pathogens. Therefore, we still have an incomplete understanding of how agricultural management systems can impact microbe-induced weed suppression.

Agricultural management may also influence the interactions between soil microbial communities and allelochemicals. For example, the allelopathy of sorghum residues is different between tillage and no-tillage systems. Tilled sorghum residue often delays the germination of following wheat crops, but no-tilled sorghum residue had little effect (Roth et al., 2000). The soil in no-tillage (Cast et al., 1990) and organic farming (Mader et al., 2002) contains a higher phenol level and more diverse allelochemical compounds than soil in conventional tillage. The rate of microbial breakdown of residue tissue may be faster than the loss rate of allelochemicals.

Many of the above examples involve one site or a few sites in a small area, but describing the treatment effects at site levels limits our ability to generalize weed biocontrol in the agroecosystem. Cross-site studies are needed for the identification of these suppression efforts that have significance on system scales. Additionally, the enormous complexity of soil microorganisms has so far limited our understanding of the relationships between agricultural management and weed suppressive microbial communities (Kennedy et al., 1996; Kennedy et al., 1995). Modern highthroughput DNA sequencing technologies offer us a way to explore the soil microbial community at higher resolution and coverage (Caporaso et al., 2012). We can understand microbial responses to agricultural management at both the community and taxon levels.

In this study, I hypothesized that different agricultural management systems can modify, to varying extents, the allelochemical and microbial weed suppressions through changing soil microbial community. Because soil in organic and no-tillage systems tends to harbor diverse and highly active microbial communities, the microbial weed suppression and microbe-residue interactions are expected to be higher in no-tillage and organic systems than tillage systems. Furthermore, I also aim to identify putative weed suppressive microbes and their responses to fresh residues in different systems. I

hypothesized that long-term management has consistent effects on weed suppressive microbes regardless of cross-site variation.

Methods and materials

Collections of fresh cover crop and soil

I selected Mammoth red clover (*Trifolium pratense*) as my model cover crop. Red clover is a widely used legume cover crop with high allelochemical potential to a wide range of weed species (Liebman et al., 2006b). I planted red clover in March 2014 and harvested whole plants at the bud stage after 14 weeks of growth. The details of the planting site can be found in Chapter 2.

To study the effects of agricultural management practices on weed suppression, I chose six agricultural fields with three types of management: organic farming, no-tillage and tillage. Two organic farms (Organic farm 1 and 2) were located in the Student Sustainable farms of University of Illinois Urbana-Champaign. The fields have been managed organically for 6 years. These fields use rotary spader which is little gentler on the soil then a traditional rototiller. These fields also use cover crops, either a rye/vetch or oats/vetch mix. Most of the plant residues are left on the soil surface and incorporated into soil before planting. The soil of these fields had pH of 7.5, organic matter 3.1% and CEC 16.4. Two no-tillage and two tillage farms were located in the Crop Sciences Research and Education Center, Urbana, IL. The soil at these sites was a mixture of Drummer silty clay loam soil and Catlin silt loam soil. No-tilled field (No-till 1) and tilled field (Till 1) had pH of 6.3, organic matter between 3.4% and 3.6%, and CEC between 15.1 and 18.5. No-tilled field (No-till 2) and tilled field (Till 2) had pH of 6.2 to 6.4, organic matter between 3.3% and 3.5%, and CEC between 20.1 and 23.4. These fields have been maintained in corn-soybean rotation for over 20 years. I sampled after a corn year for all tillage and no-tilled fields.

I collected soil from these sites in June 2014 to a depth of 10 cm. I collected bulk soil samples and stored them in a sealed plastic bucket at 4 ℃ for up to 1 week before use. I divided this soil into two portions. One portion stayed at 4 ℃ to keep fresh; another portion was triple-autoclaved at 120 ℃ for 1 h to kill soil microorganisms. Because autoclaving releases microbial biomass nitrogen and reduces soil moisture, after

autoclaving, I measured the gravimetric soil moisture, KCl-extractable ammonium and nitrate of sterilized and unsterilized soil. I adjusted the soil moisture, soil ammonium and nitrate of the sterilized soil to the same as that of live unsterilized soil.

Experimental design

I conducted a fully-factorial mesocosm experiment (live vs. sterilized soil and fresh residue vs. no residue) to test the effects of fresh residues and microbes on weed germination and growth. I added fresh red clover residues to the soil to mimic a typical "green manure" management strategy. Fresh red clover was cut into 5-cm pieces and completely dispersed in Zip lock bags. To control the introduction of microorganisms present in the plant tissues, fresh residues were sterilized by UV light for 2h on each side of the plant tissue prior to addition to the mesocosms (Wilson et al., 1999). I added 2% (by weight) of fresh red clover residues to the soil, because this percentage is similar to field incorporation rates of red clover (Dyck et al., 1995) and has sufficient germination suppression on mustard seeds (Liebman et al., 2006a). Each mesocosm contained 110 g of soil. Therefore, each mesocosm contained (110 g x 2 % =) 2.2 g of fresh red clover residue. Mesocosms were fitted with lids with a filter-covered hole to maintain sterile conditions and minimize water loss.

To understand how weed suppression changed with time after residue incorporation, I assayed the weed suppression potential of mesocosms at different times. I set up all mesocosms on the same day, and then I conducted these assays at days 0, 5, 10, 20 and 40 after residue incorporation. At each of these time points, I randomly selected 3 replicate mesocosms from each of the 2 microbe x 2 residue treatments x 6 fields (total 72 mesocosms), collected 10g soil into separate centrifuge tubes, and stored them at -20 ℃ for analysis of soil phenolic content (see below). I used the remaining 100g soil in each mesocosm to conduct the bioassays described below. Mesocosms were arranged in the glasshouse according to a fully randomized design, and I re-randomized the placement of the remaining mesocosm after each assay time point.

Bioassay of seed germination and growth

I used a seed bioassay method modified from Dabney and colleagues (1996) to assess the effects of microbes and fresh residues on weed germination and growth. I chose IdaGold wild mustard (*Sinapis alba*) as the target weed species, because it is a common weed in temperate agroecosystems, and this variety has a very high and uniform germination rate based on the trial test. Therefore, the influence of seed dormancy on the estimation of seed germination was minimized.

I constructed one bioassay from one mesocosm. I moistened one layer of 25 cm by 38 cm germination paper (Anchor Paper, St. Paul, MN) with 20 ml of sterilized, deionized water. Then I lined up 15 mustard seeds 10 cm from the top edge of the germination paper. I spread the 100g soil from a mesocosm in a 12cm wide band, about 6 cm from the top edge of germination paper, to cover the line of seeds. The second pre-moistened sheet of paper was placed on the top the seeds and soil. I rolled this entire assembly from the short edge and sealed in Zip-lock bag to maintain soil moisture content. I incubated these bioassay units vertically with seeds oriented "up" in the upright cylinder in a Conviron 125-L incubator (Controlled Environments Limited, Manitoba, Canada) for 7 days with a 16 h light: 8 h dark cycle (25 ℃ and 20 ℃, respectively).

After 7 days of incubation, I deconstructed each bioassay unit and recorded the number of germinated seeds and the seedling length of all germinated seedlings. I also recorded the number of seedlings with visible necrotic lesions and the length of visible necrotic lesions, which I considered to be disease incidences on seedlings for the purposes of this study.

Soil total phenolics content

I collected soil from mesocosm at the time that each bioassay was setup. I used total phenolic compounds as a proxy for plant-derived allelochemicals (Inderjit, 1996; Ohno et al., 2000). The extraction process was same as Chapter 2. I quantified phenolics by using the Folin-Ciocalteu method (Ainsworth et al., 2007). I mixed 0.1 mL of extract, 0.2 mL of 1:10 diluted Folin–Ciocalteu's phenol reagent and 0.8 mL of 700nm sodium carbonate and incubated the mixture for 2 h at 23 ℃. I then measured absorbance of the

mixture at 765 nm on a microplate reader (Biotek Instruments, Inc.) and used gallic acid standards to create a standard curve.

HPLC analysis of allelochemical compounds

Isoflavones are the main allelopathic compounds in red clover (Macias et al., 2007). I used HPLC to analyze 12 main isoflavones in red clover (Krenn et al., 2002). They are biochanin A, calycosin, daidzein, daidzin, formononetin, genistein, genistin, glycitein, prunetin, quercetin, quercetion and kaempferol. Samples were analyzed with LC/MS/MS /HPLC system in Metabolomics Center, University of Illinois. The analysis procedure was the same as Chapter 2.

DNA extraction of soil microbes on dead seeds and live seedlings

For each bioassay, I collected samples from three soil fractions in "live" soil treatments: dead seeds, diseased live seedlings, and soil, to characterize total soil bacterial and fungal communities. Soil microbial DNA extractions used the FastDNA SPIN kit for Soil, and DNA extraction of microbes on seeds and seedlings used regular FastDNA SPIN kit (MP Biomedicals, Solon, OH), followed by a purification with 1% cetyltrimethylammonium bromide and a chloroform:isoamyl alcohol extraction to remove residual soil impurities (e.g. humic acids).

Illumina sequencing

The bacterial community was accessed by sequencing the V3 – V4 region of 16S rDNA using the PCR primers 515F and 926R (Caporaso et al., 2011; Lane, 1991). The fungal community was accessed by sequencing the ITS2 region of ITS using the PCR primers ITS3 and ITS4 (White et al., 1990). The details of Illumina sequencing can be found in my previous study (Chapter 3).

Sequence pre-processing

Paired-end raw sequence reads were merged with the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Merged sequences were filtered by requirements that the minimum quality score >30 and 95% percent of bases must have 30 quality score. USEARCH version 8.0.1517 (Edgar, 2010) was used to do the following processes. Sequences were sorted by abundance and then singletons were removed. The

clustering procedure at 97% similarity threshold within USEARCH (UCLUST) was performed using the default parameters. Representative sequences for each OTU were obtained from USEARCH. USEARCH/UCHIME was used to detect Chimera using Gold database (Kyrpides, 1999) as reference for bacteria and UNITE ITS database for fungi (Abarenkov et al., 2010). The representative sequences of bacteria were aligned and assigned taxonomic information by Greengenes database within QIIME (DeSantis et al., 2006). Fungal ITS2 sequences were too diverse to be aligned. The taxonomic information of fungi sequences were directly assigned by QIIME using UNITE ITS database (Abarenkov et al., 2010).

Data Analyses

For each 15-seed bioassay unit, I calculated the following values: percentage of germinated seeds, mean length of seedling, percentage of diseased seedlings (the number of seedlings with visible necrotic lesions divided by the number of germinated seedlings), and disease severity (the length of visible necrotic lesions divided by the total length of germinated seedlings). I used a nested model to test the effects of management, microbe, residue fraction, and time on percentage of germination and seedling length. The nested model had management, microbe, residue fraction, and time as fixed effects, and field as random effects nested with management. Within each combination of residue and microbes treatment, I conducted Tukey's HSD test to determine whether the percentage of germination and seedling length were different among management systems. The nested models were analyzed by “lme” functions from package “nlme” in R.

I also sought to quantify the relative contributions of microbes, fresh residues, and their interactions to the weed suppressive effects in different fields at different time points. I considered two different dimensions of weed suppression of soils: Germination Inhibition (GI) and Seedling Length Inhibition (SI). The details of GI and SI were explained in Chapter2. To exemplify, the calculations for the various components of GI are as follows:

$$\text{Microbe-only\_GI} = G_{\text{sterile soil + water-only}} - G_{\text{live soil + water-only}}$$

$$\text{Residue-only\_GI} = G_{\text{sterile soil + water-only}} - G_{\text{sterile soil + residue}}$$

Interaction_GI = $G_{\text{sterile soil + water-only}}$ - $G_{\text{live soil + residue}}$ - Microbe-only_GI - Residue-only_GI

I used linear regression to determine whether the disease severity can affect seedling growth. I performed analyses separately for soil with and without fresh residue in each management to determine the whether the effects of disease severity were different between fresh residue treatment and management systems.

To explore overall patterns in chemical composition, I used Principal Components Analysis ordination. Seed germination, seedling length, percentage of diseased seedling, disease severity, and days were fitted on the ordination to explore their correlation with chemical composition ordination. All of these analyses used functions from package "vegan" in R (Oksanen et al., 2009).

Microbial community analyses

I used non-parametric permutational multivariate analysis of variance (Permutation MANOVA) to analyze the effects of soil fraction (dead seed, seedling and soil), residue addition, day, agricultural system and field on microbial communities. This analysis used function "adonis" in package "vegan" in R (Oksanen et al., 2009). I used ANOVA and Tukey-HSD to test the effects of treatments on the diversity of microbial communities.

Because putative weed suppressive organisms are likely to be present on dead seeds and diseased seedlings (as opposed to healthy seedlings), I used "Indicator species analysis" (package "indicspecies" in R) to find OTUs that were enriched on dead seeds and diseased live seedlings comparing to the soil. The indicator species analysis determines the strength of the association between a microbial OTU and an environment condition. It considers the relative frequency and abundance of microbes in the target versus non-target environment (De Caceres et al., 2009). Any OTU with a significant ($p < 0.05$) indicator value was considered as enriched OTU on dead seeds (or diseased seedlings). I performed analyses separately for each field.

I used network analyses to determine the correlation between microbial OTUs and to investigate whether the correlated OTUs acted differently between systems. I only used microbial OTUs that have been considered as indicator species on dead seeds and infected live seedlings. I firstly tested the non-random co-occurrence patterns with the

checkerboard score (C-score) under a null model preserving site frequencies (Bailey et al., 2003). I considered it to be a valid co-occurrence between OTUs if the Spearman's correlation coefficient (r) was both > 0.6 and statistically significant (P-value <0.01) (Jilani et al., 2008). I used a bipartite association network to visualize the associations between OTUs and the different agricultural managements. Statistical analyses and visualization were carried out in the R in package "igraphy" (Walters et al., 2010).

I am particularly interested in microbes that can be stimulated or depressed by the addition of fresh residues. Thus, I did a further selection on microbes that were identified by indicator species analysis. I conducted t-test for each OTUs to determine whether the abundance of that OTU significantly changed between residue treatment and water control treatment (P-value<0.05).

Results

Effects of fresh residue, microbes and interactions on weed suppression

The main effects of fresh residue, day, and microbe on seed germination and seedling growth were all significant. The main effect of management was not significant, but its interactions with other treatments were all significant, except for the interaction between day and management on seedling growth (Table 4.1). Microbes demonstrated constant suppression on seed germination and seedling growth without the presence of fresh residue. The microbial suppression was lowest in no-till management but was not different between organic and tillage management systems (Tukey's HSD test) (Figure 4.1). However, microbial communities also reduced the suppression from fresh residues. In soil amended with fresh residues, weed germination was significantly higher in live soil than sterile soil (T-test, P-value<0.05 for three management systems). Effects of microbial communities and fresh residue on seedling length were generally similar to the results of seed germination (Figure C.1).

The relative strengths of three suppression sources, microbe-only suppression, residue-only suppression, and their interaction, varied dynamically over time and across the three management systems (Figure 4.2). Microbe-only inhibition of germination (GI) and seedling growth (SI) was relatively stable within fields over time (Figures 4.2 and

C.2). The residue-only GI was consistently higher than microbial-only GI over the entire experimental period. The residue-only GI was stable in the organic management system but declined over time in tillage and no-tillage management systems. The microbe-by-residue interaction was always negative for all management systems over the course of the experiment. Generally, the negative microbial-by-residue interaction increased over time and exceeded microbial-only suppression at day 40. The temporal pattern of microbe-only SI was generally similar to that of GI (Figure C.2).

Disease incidences on seedlings

Without allelochemicals, the percentage of diseased seedlings and disease severity significantly inhibited seedling growth in live soil with water (P-value<0.05) (Figure C.3). The average seedling length was shorter in the organic system (71.4mm) and tillage (72.5mm) system than the no-tillage (117.3mm) system (T-test, P-value<0.05). But the negative effects of disease severity on seedling growth were strongest in no-tillage system (-98.07), following by the organic system (-87.27) and till system (-64.5). When allelochemicals were present, the disease severity did not influence seedling growth.

Phenolic weed suppression

Overall, seed germination and seedling length decreased as total soil phenolic content increased (Figure C.4). Without microbes, a phenolic content higher than 8 ng/g can completely suppress weed germination in organic soils. At least 16 ng/g soil and 20 ng/g soil were needed to completely suppress weed germination in tilled and no-tilled soil, respectively. Microbial weed suppression depended on soil phenolic content (Figure C.4). When phenol was lower than 8 ng/g soil, the presence of a live microbial community resulted in lower germination percentage than sterilized soil with similar phenol levels. However, in most of cases, when phenol was higher than 8ng/g soil, the presence of a live microbial community resulted in higher seed germination than sterilized soil.

Allelochemical composition and dynamics

Agricultural management systems had great impacts on fresh residue released allelochemical composition and dynamics. Overall, germination percentage, seedling growth, and day were all negatively correlated with the allelochemical compounds

(Figure 4.3). Organic and tilled fields had higher contents of allelochemicals in soil than no-till fields, but they all quickly reduced to the similar level as the water control after 20 days (Figure C.5).

Total microbial community

Sequence clustering yielded 4649 bacterial and 2107 fungal OTUs in total. Agricultural management and soil fraction (seed, seedling, and soil) were the most influential factors on microbial community compositions. These two variables explained the 27% variance in bacteria communities and 21% variance in fungal communities. The variance explained by the residue and day were smaller than the above two factors but still significant (Table 4.2).

The diversity of bacterial communities in organic system was significantly higher than in the conventional (tilled) system (P-value<0.05). The diversity of fungal communities was highest in organic system, but the difference was not statistically significantly.

Microbes enriched on dead seed and diseased seedling

The whole microbial communities on dead seeds and diseased live seedlings were significantly different (Permutational MANOVA, P-value<0.001 for both bacteria and fungi). 156 bacterial OTUs and 90 fungal OTUs were enriched on dead seeds and diseased seedlings. The bacterial OTUs enriched on dead seeds were mostly in the phyla *Firmicutes* (45%) and *Proteobacteria* (29%), *Bacteroidetes* (21%), and OTUs enriched on seedlings were mostly in the phyla *Proteobacteria* (50%) *Bacteroidetes* (18%) and *Firmicutes* (9%) (Figure 4a). The fungal OTUs enriched on dead seeds were mostly in the order *Mucorales* (46%) and class *Sordariomycetes* (31%). OTUs enriched on seedlings were mostly unidentified (37%) and in class *Sordariomycetes* (21%) (Figure 4.4b).

The composition of microbes enriched on dead seeds and diseased live seedlings was also influenced by agricultural management (Permutational MANOVA, P-value<0.01 for both bacteria and fungi). There was also apparent field to field variation of microbial communities within management types (Figure 4.4). However, the microbes from two fields under the same management always had more common OTUs than that

from different managements (Table C.1). Moreover, microbes enriched on dead seeds had more common OTUs than microbes enriched on diseased living seedlings.

The co-occurrences networks of OTUs enriched on dead seeds and diseased live seedlings were markedly different (Figure 4.5). Both networks were significantly different with non-random co-occurrence patterns (P-value<0.01). In general, bacteria dominated the network of dead seeds while the network of diseased seedlings was dominated by fungi. The network of dead seed was smaller and simpler than the network of diseased seedling. The network of dead seeds had 57 nodes, 3.0 mean degree of connections, and the modularity was 0.72 with 10 modules while, for the seedlings, the network had 127 nodes, 4.0 mean degree of connections, and the modularity was 0.51 with 15 modules. For these microbial OTUs in the network, most of them (dead seeds: 60% and diseased live seedlings: 62%) were identified as enriched species in multiple management systems. OTUs from the organic system had the highest number of connections (dead seed: 55% and diseased live seedling: 78%).

Microbes that were stimulated or depressed by fresh residues

In the following, I focus only on OTUs that differed significantly between the residue treatment and water treatment. In total, 55% bacterial OTUs and 16% fungal OTUs changed significantly with the addition of fresh residues in all the fields. Overall, the magnitude of increase was significantly larger than the magnitude of decrease (T-test, P-value<0.01) (Figure 4.6). The majority of decreased bacterial OTUs were in the phyla *Proteobacteria* (72%) and *Bacteroidetes* (15%). However, *Proteobacteria* also had the highest percentage (53%) of all increased abundance. The majority of decreased fungal OTUs were in the class *Sordariomycetes* (38%) and the order *Mortierella* (31%). The increased fungal OTUs mostly belonged to *Agaricomycetes* (78%). The changes of microbial relative abundance varied among fields (ANOVA, P-value<0.05). OTUs in organic farms were least likely to be depressed by the residue addition (TukeyHSD, P-value<0.05).

Discussion

This study demonstrated that agricultural management systems had strong impacts on microbial communities (Table 4.2), which lead to various fresh residue-microbial interactive effects on weed suppression potentials over time (Figure 4.1 and figure 4.2). Putative weed suppressive microbes and their responses to green manure were heterogeneously distributed across the different types of management. However, some consistent patterns were observed, for example, more shared putative weed-suppressive microbes within management system than between systems. (Table 4.1). My results suggest that human management can alter soil microbial communities in spite of the field-to-field variance. This supports the notion that proper management of the soil microbial communities can generate desirable weed suppression.

Agricultural management alters microbial weed suppression

Since many studies have already shown that organic and no-tillage systems have various positive effects on the belowground biota (Flohre et al., 2011; Kandeler et al., 1999; Mader et al., 2002; Moeskops et al., 2010), I hypothesized that microbial communities in organic and no-tillage systems would have stronger microbial weed suppression than till system. My results support the hypothesis for the microbial weed suppression in organic system, but not the no-tillage system (Figures 4.1-4.2). Without red clover-derived allelochemicals, the organic and tillage systems had stronger microbial suppression on germination (Figure 4.1) and seedling growth (Figure C.1), and more diseased seedlings than the no-tillage system (Figure C.3). These observations are supported by the discoveries from microbial data. The microbial diversity in organic and tillage systems was higher than in the no-tillage system. This high taxonomic diversity has the potential to harbor more pathogens. Beside the microbial diversity, putative weed-suppressive OTUs from organic systems had the highest number of connections within themselves and also with OTUs from other systems (Figure 4.5). Microbes in organic farms seem most correlated with microbes from other systems. One possible reason of this co-concurrence pattern is that organic compost amendment activates diverse groups of microorganisms (Barberan et al., 2012; Mader et al., 2002). The complexity of organic substrates encourages microbial generalists with broad ecological niches and interaction

possibilities. It is also possible that that tillage is the important management component that caused the patterns. The tillage intensity of organic farms in this study is intermediate between conventional tillage and no-tillage, thus the microbes may have similar responses to microbes in either tillage or no-tillage systems. Because it has been observed that taxonomic diversity and synergistic performances are positively correlated with the pathogenicity of fungal pathogens (Chandramohan et al., 2001; Chandramohan et al., 2002), these two characteristics may be important for the high weed suppression in organic system. But it is difficult to tell which one is truly important because all the factors are confounded.

Agricultural management alters microbes- fresh residue interactions

Among these three management systems, the organic system in this study demonstrated the strongest and most persistent suppressive potential with and without microbes (Figures 4.1-4.2). This pattern is consistent with the observation that the highest soil allelochemical levels were present in the organic fields until day 10 (Figure C.5). The allelochemicals were dominated by the high potent phytotoxic chemicals formononetin (Liu et al., 2013) and biochanin A (Shajib, 2012), which were negatively correlated with germination and seedling growth (Figure 4.3). In the absence of microbes, the allelochemicals increased in organic fields until day 5 – day 10 (Figure C.5). This suggests that the soil was better in accumulating allelochemicals than losing them. Organic management systems incorporate diverse organic substrates in the soil, which increase the soil organic matter content (Wander et al., 1996). High soil organic matter may improve the retention and protection of allelochemicals (Cheng, 1992; Dalton, 1999). In the presence of microbes, the overall concentrations of allelochemicals was higher in tillage and organic systems than in the no-tillage system, but the dynamics of allelochemicals varied a lot from field to field (Figure C.5). This large variability may be related to the different soil properties in these fields.

The microbe-by-residue interaction was always negative throughout the experiment (Figure 4.2). I found that some putative weed-suppressive microbes were depressed by the addition of red clover residues (Figure 4.6). One possible reason of negative effect is that the phenolic compounds in red clover are produced for defense against pathogens (Dakora et al., 1996; Inderjit, 1996). Another reason may be the

oligotrophic life styles of these microbial taxa, which may be disfavored when carbon-rich plant residues are incorporated. For example, all the OTUs in *Acidobacteria* were depressed. Some studies suggest members of these phyla are oligotrophs that prefer a nutrient-limited environment (Bastida et al., 2013; Fierer et al., 2007). They may be out-competed by copiotrophic microbes in this carbon rich environment.

Although the organic and tilled soil both contained a large amount of allelochemicals, the weed-suppressive microbes in organic soils had the smallest decreased abundance (Figure 4.6). These results indicate that the weed suppressive microbes in organic soil were less sensitive to the negative effects of allelochemicals than microbes in other two systems. There is evidence that high concentration of phytotoxic compounds can induce toxicity tolerance in fungal pathogens (Morrissey et al., 1999). Therefore, as a next step, it would be interesting to evaluate how the weed-suppressive microbes from organic farms respond to the different concentrations of allelochemicals compared to the microbial communities from other systems.

Putative weed suppressive microbes

Because of the tremendous diversity of soil microbial communities and their responses to the soil management, it is not surprising to find that weed-suppressive microbes and their responses varied a lot from field to field in this study. This significant field-to-field variability is common to field studies conducted in similar crop systems (Davis et al., 2006; Hartmann et al., 2015). Despite this variability, I found some consistent patterns underlying the weed-suppressive microbes across the treatments.

One common response of the three management systems is that fungi dominated the communities enriched on diseased seedlings, and bacteria dominated the communities enriched on dead seeds (Figure 4.5). One possible reason of this finding is that fungi and bacteria have different speeds of response to the seed germination process. Studies found that the peak of carbohydrates exuded from pea seeds occurs with 10 hours after sowing (Gorecki et al., 1985; Short et al., 1976). Pathogens that can respond rapidly on this time scale may be more competitive in colonizing seeds (McKellar et al., 2003). Because bacteria are motile and fast-growing, while fungi are nonmotile and slow-growing, bacterial pathogens may respond to the seed exudates quicker than fungal pathogens. However, fungi have the advantage of hyphal growth to penetrate vascular plant tissue,

which probably helps fungi to infect a live seedling (de Boer et al., 2005). This result suggests a shift from bacteria to fungi in weed-suppressive communities as the seed germinates.

The highthroughput sequencing approach provides the potential to identify microbial taxa responsible for microorganism-derived weed suppression. Among the 90 fungal OTUs enriched on diseased live seedlings, 50 of them were assigned at genus level. 25% of the genera are *Fusarium*, *Rhizoctonia solani*, *Pyrenochaeta,* and *Mucor*. Species of these genera have been known as pathogens (Banuett, 1995; Kremer et al., 1996; Nordskog et al., 2008) or endophytes of plants (Cloete et al., 2011; Kremer et al., 1990). Some fungal species, like *Pleosporales.sp*, *Sporormiaceae.sp* and *Mortierella.sp,* live as saprotrophs on rotting roots, decaying leaves and other organic material (Oyarzun et al., 1998). About 13% of bacterial OTUs enriched by dead seeds were members of the genera *Achromobacter, Pseudomonas, Enterobacter, Pantoea*, *Flavobacterium,* and *Xanthomonas*. Species in *Flavobacterium* and *Pseudomonas* genera have been found as deleterious rhizobacteria (DRB) of plants (Kremer et al., 1990; Nehl et al., 1997). *Pseudomonas syringae* is a common foliar bacteria responsible for many important plant diseases (Whalen et al., 1991). *Enterobacter* can produce phytotoxins to inhibit root elongation (Schroth, 1986). *Xanthomonas campestris* cause bacterial spot disease of various brassica plants (Vicente et al., 2013).

For these microbial taxa that were associated with weed-suppressive activities and enriched by the addition of residues, I find that 176 bacterial taxa and 24 fungal taxa identified as weed-suppressive in Chapter 3 were similarly identified as weed-suppressive by the analyses in this current chapter (Table C.2). These microbial taxa were consistently highly correlated with weed-suppressive activities among different types of soil, confirming their importance in suppressing seed germination or seedling growth. Some of these microbial taxa are known plant pathogens, such as *Fusarium* and *Pseudomonas* (Charudattan, 2001; Ploetz, 2006; Whalen et al., 1991). However, most of them have little information on taxonomic classifications and interactions with plants. It is possible they are novel pathogens that have not been fully characterized yet. Future investigation is needed to gather the fundamental knowledge about them. Even for these known pathogens, I can only speculate on the ecological roles of the microbial taxa based

on previous information. More study is needed on whether these microorganisms can be isolated and re-inoculated, and whether they can persist to produce weed-suppressive effects in the soil.

Conclusion

Agricultural soils under different management practices have various weed suppression potentials. These variances were notable among replicated field plots but smaller than the management-induced differences. Overall, organic and tillage systems offered higher microbe-derived and fresh residue-derived weed suppression than the no-till system. Soil microbes in these two systems were more effective in pathogenic suppression and acceleration of allelochemical release from fresh residues. A deeper investigation of the putative weed-suppressive microbes suggests that distinct communities were associated with seed germination suppression and seedling growth suppression. Putative weed-suppressive microbes from organic systems were less sensitive to the phytotoxic effects of fresh residues and more connected among individual taxa than tillage and no-tillage systems. The specific differentiation at the level of individual microbial taxa in this study offers novel insights into the potential of managing the soil microbiome for sustainable weed control strategies.

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Tables and figures

Table 4.1. Nested models showing the treatment effects on percentage of germination and seedling length.

	Percentage of germination		Seedling length	
	P-value		P-value	
Intercept	<.0001	***	<.0001	***
Residue	<.0001	***	<.0001	***
Day	<.0001	***	<.0001	***
Microbe	<.0001	***	<.0001	***
Management	0.0915		0.4467	
Residue : Day	<.0001	***	0.001	**
Residue : Microbe	<.0001	***	<.0001	***
Day : Microbe	0.0001	***	0.2172	***
Residue : Management	0.0004	***	0.1978	***
Day : Management	0.0006	***	0.7546	
Microbe : Management	0.0002	***	<.0001	***
Residue : Day : Microbe	0.0004	***	0.2015	
Residue : Day : Management	0.0006	***	0.0387	**
Residue : Microbe : Management	0.0008	***	0.0014	***
Day : Microbe : Management	0.0222	**	0.4137	
Residue : Day : Microbe : Management	0.1295		0.996	

Significance codes: alpha <0.001: ***, alpha <0.01: **

Table 4.2. Permutational MANOVA showing the treatment effects on soil microbial community composition.

Bacterial community	R2	P-value	
Residue addition	0.03962	0.001	***
Day	0.02712	0.001	***
Soil fraction (seed, seedling and soil)	0.1796	0.001	***
management	0.09796	0.001	***
Fungal community	R2	P-value	
Residue addition	0.03334	0.001	***
Day	0.02739	0.001	***
Soil fraction (seed, seedling and soil)	0.12941	0.001	***
management	0.07815	0.001	***

Significance codes: alpha <0.001: ***, alpha <0.01: **

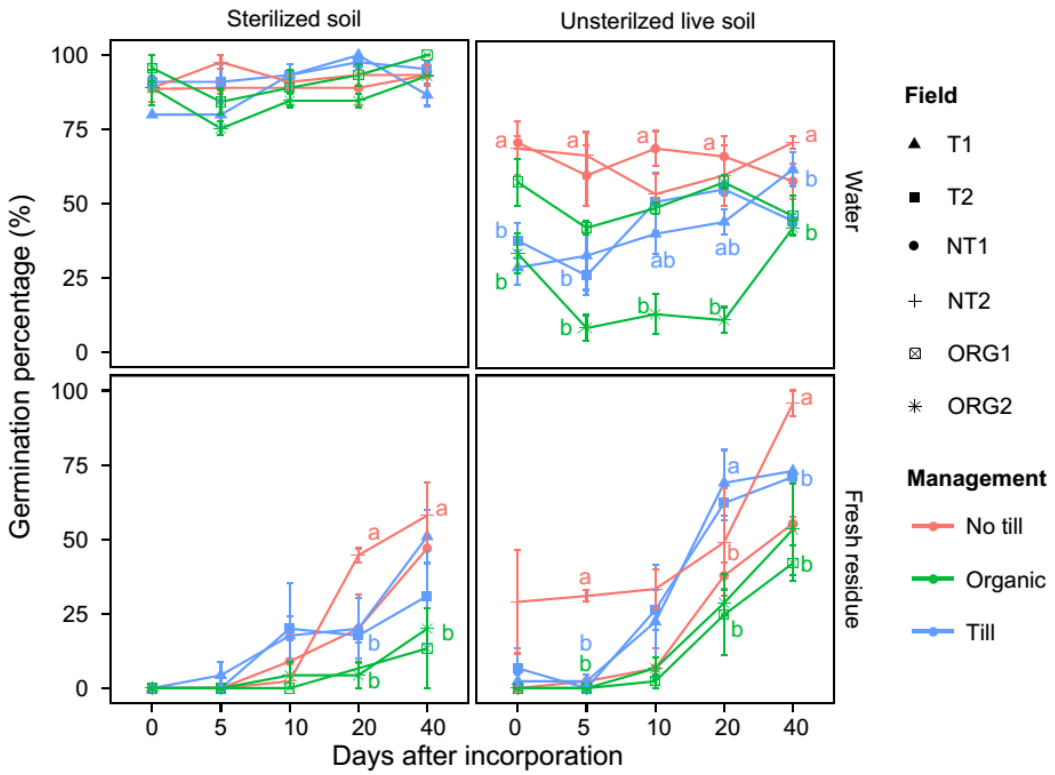

Figure 4.1. Seedling germination of mustard with water or fresh residues in sterilized soil and live soil. Standard error from three replicate analyses are shown. Letter a-b indicate significant difference among management systems at $P < 0.05$ by a Tukey's HSD test. The color of letter indicates the management system.

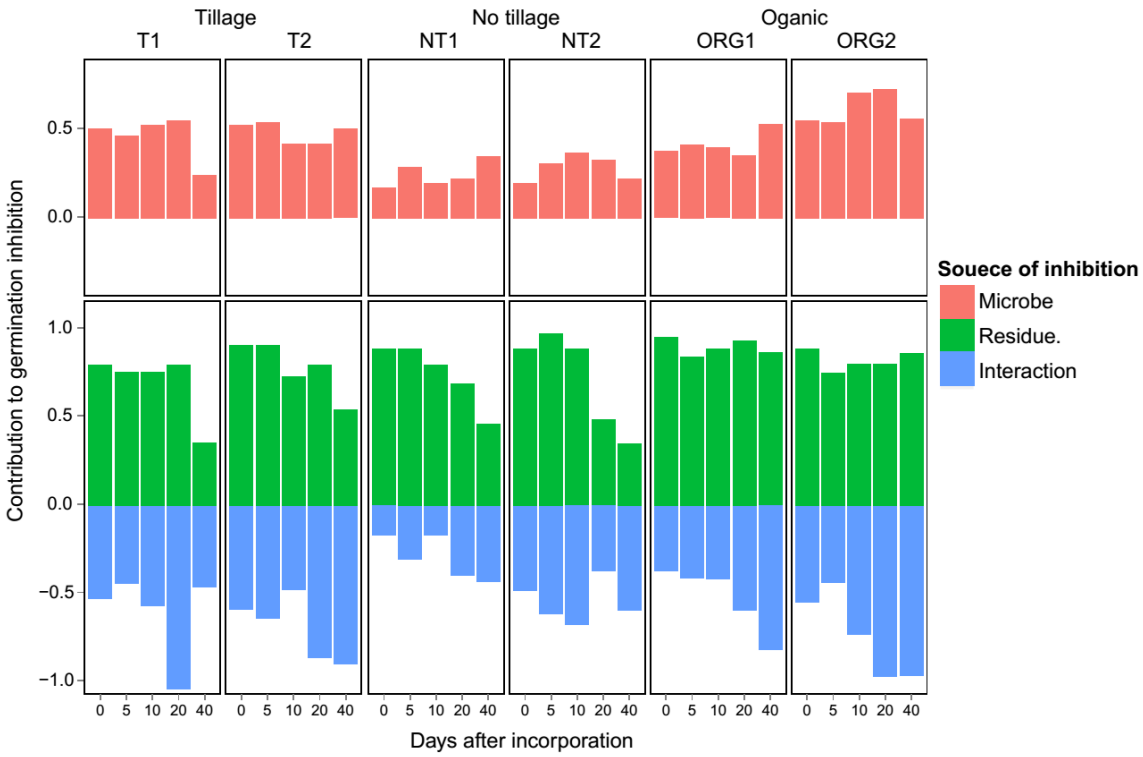

Figure 4.2. Antagonistic interactions between soil microorganisms and red clover residues differ between agricultural systems. Bars indicate the strength of microbe-only, residue-only, and microbe by-residue contributions to germination inhibition.

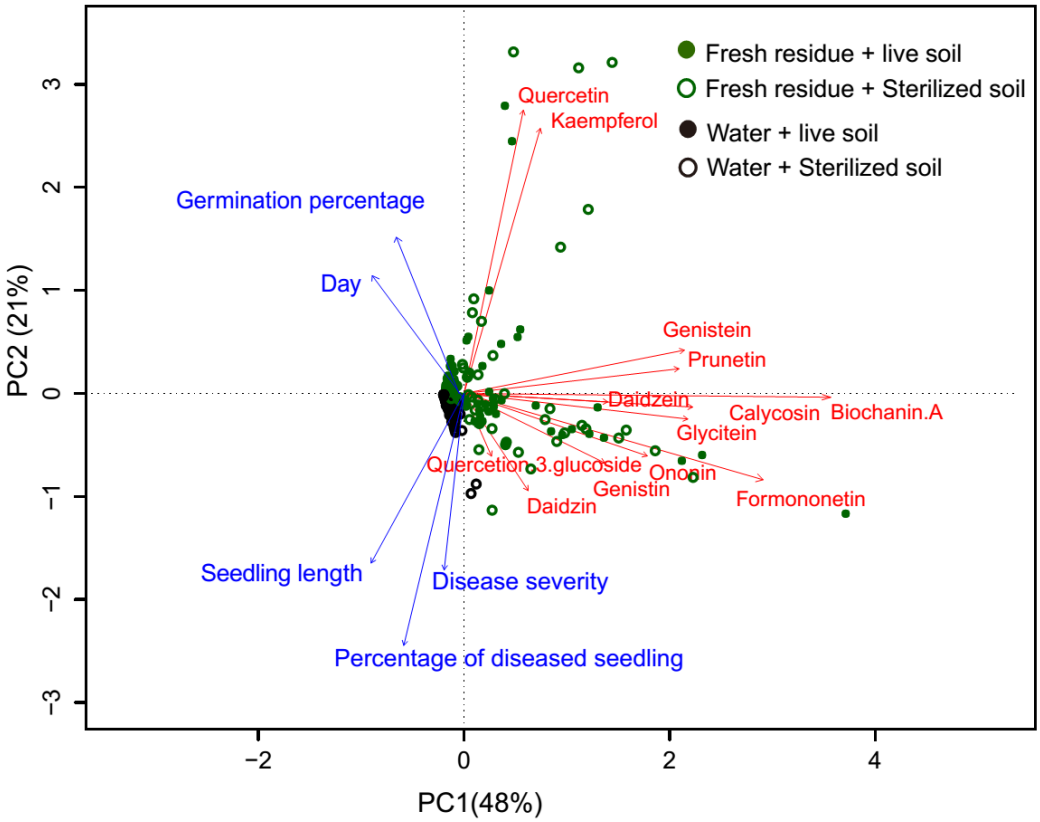

Figure 4.3. Principal component plot of twelve allelochemicals fitted with weed germination, seedling length, percentage of diseased seedlings, disease severity of seedlings, and day.

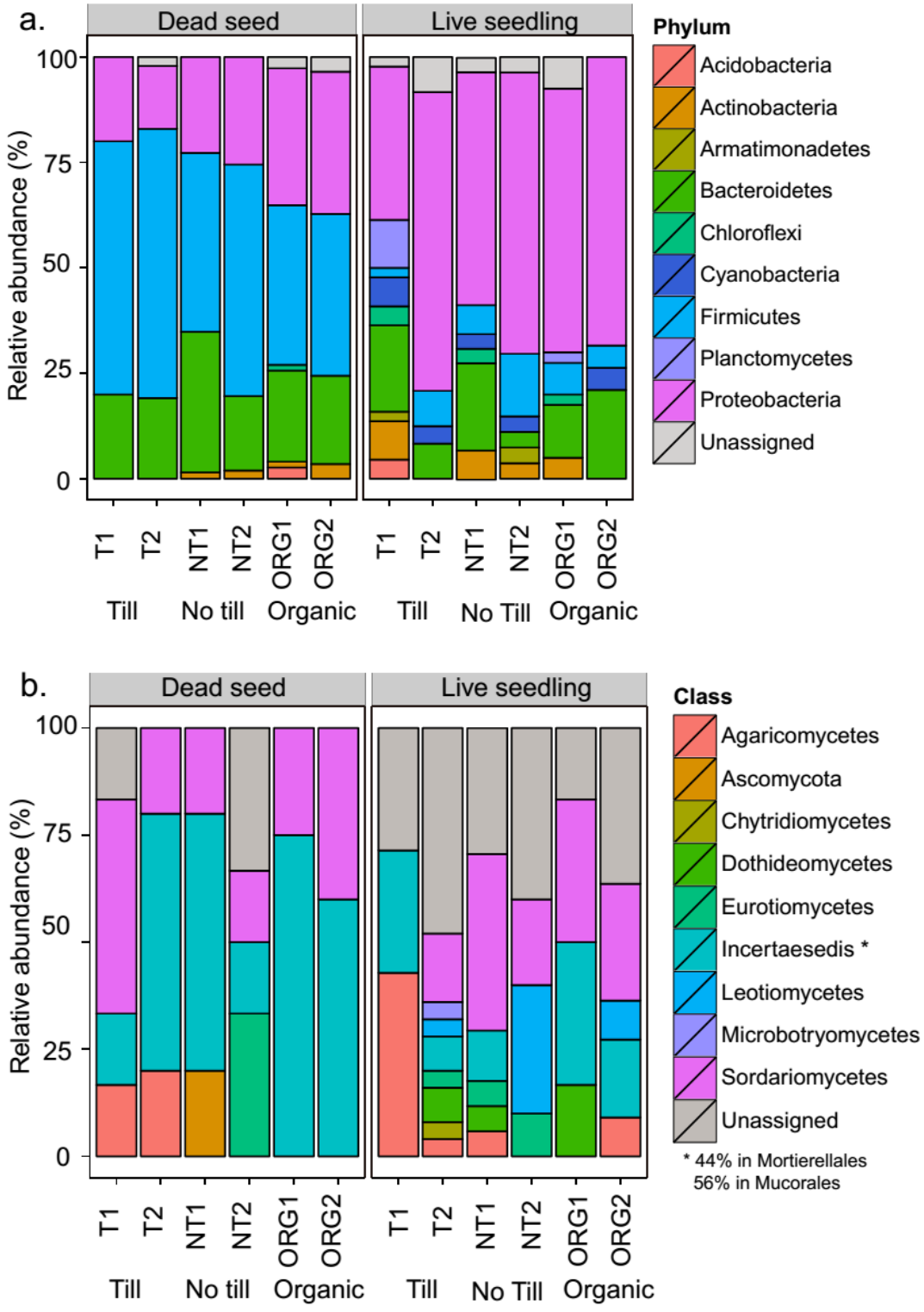

Figure 4.4. Relative abundance of major (a) bacterial phyla and (b) fungal classes that were enriched on dead seeds and diseased live seedlings.

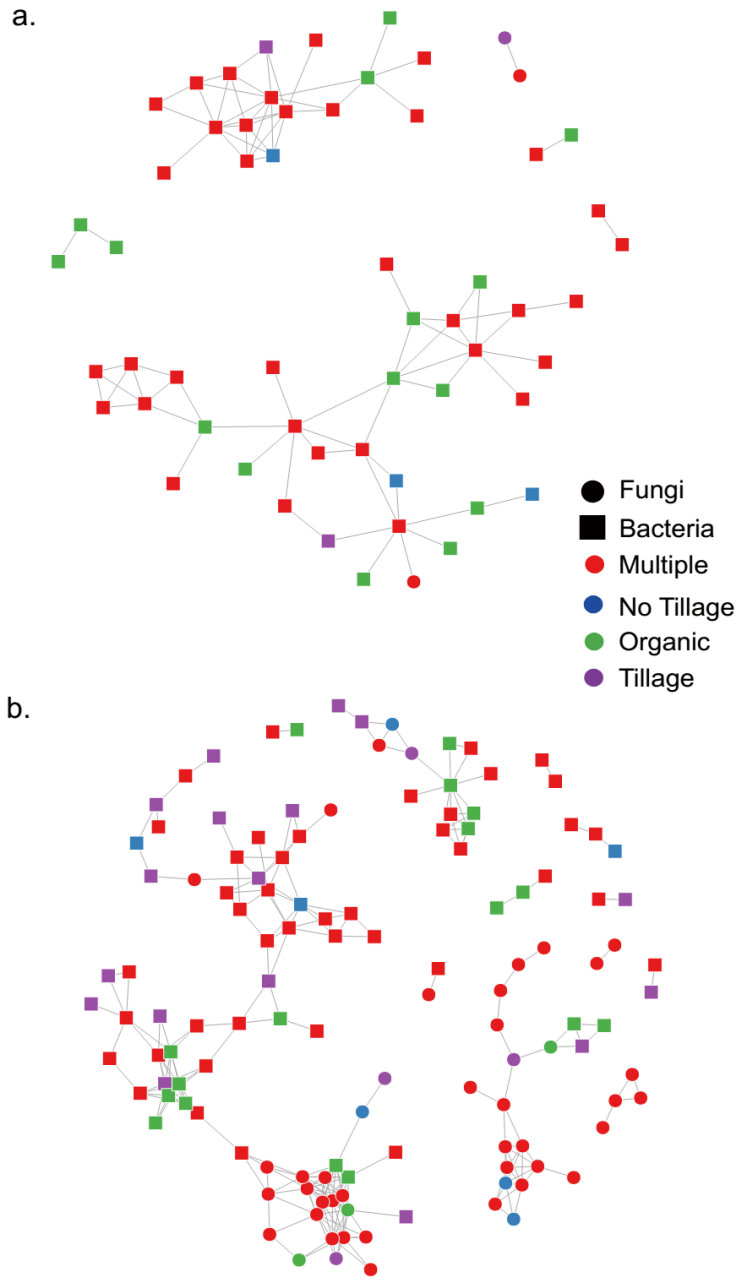

Figure 4.5. Co-occurrence networks of bacterial and fungal communities that were enriched on (a) dead seeds and (b) diseased live seedlings. The colors of OTUs represent the management system where OTUs were identified as enriched species. Multiple: microbes were identified as enriched species in more than one management systems.

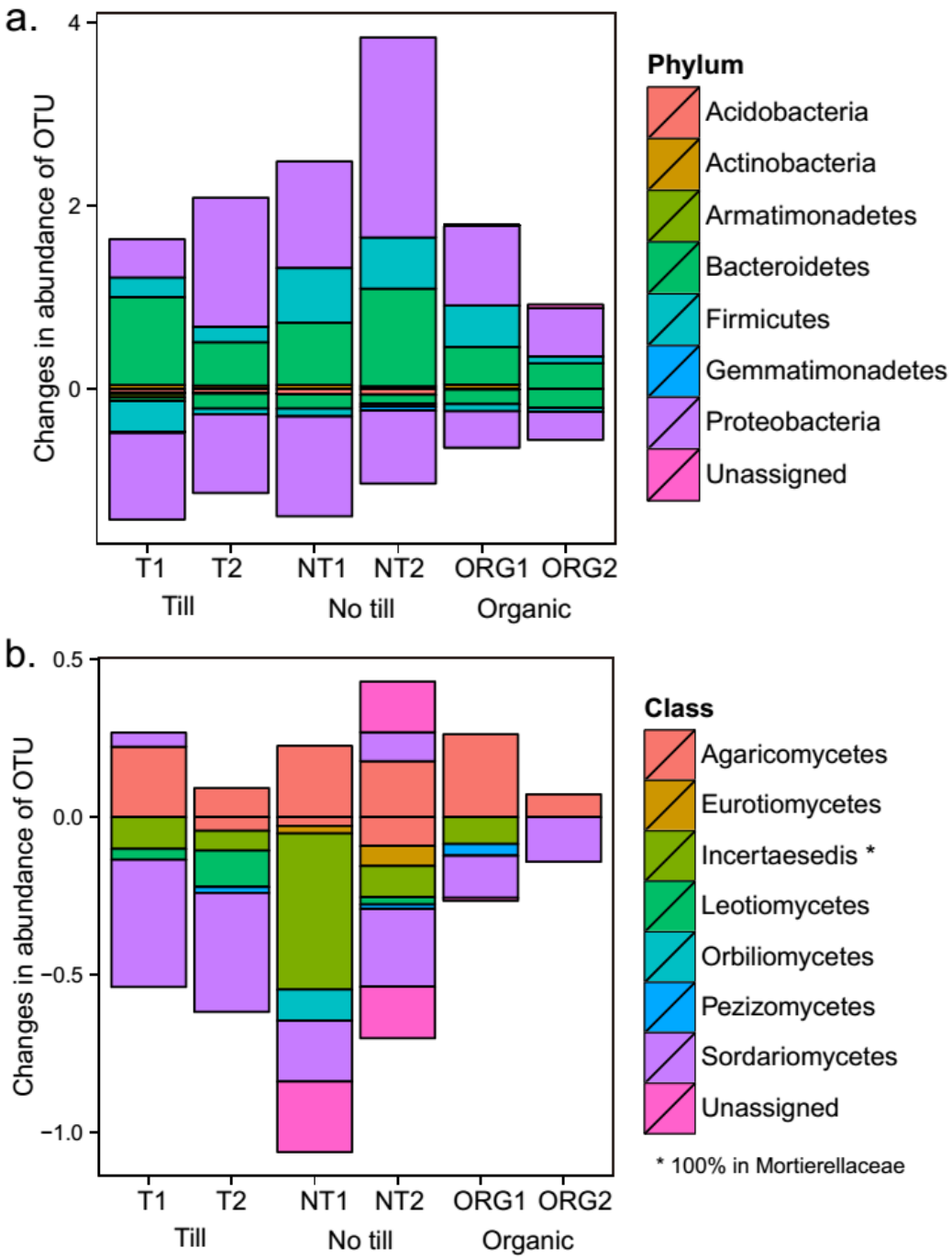

Figure 4.6. Composition of major (a) bacteria phyla and (b) fungal classes that were significantly changed by the addition of fresh residues. Only microbial OTUs that were enriched on dead seeds and diseased seedlings were included.

CHAPTER 5: EFFECTS OF ALLELOCHEMICALS FROM COVER CROP RESIDUE ON WEED SEEDLING DISEASE

Abstract

Soil allelochemicals can strongly influence microbial communities, consequently influencing microbial weed suppression. However, both positive and negative effects of allelochemicals on microbial attacks on plants have been observed before. Many allelochemicals have anti-microbial properties, which may inhibit seedling disease. The allelochemicals can also damage the seedling membrane and induce seedling leakage. The leakage induced by the allelochemicals may make the seedlings more vulnerable to microbial attack by attracting chemotactic microbes. In this study, I conducted a series of experiments to test the effects of water-soluble extracts of red cover residues, biochanin A and formononetin on seedling disease. I tested the microbial responses to the seedling leakage to determine their chemotactic behaviors, and I also identified these microbes by sequencing. Bioassay experiments demonstrated that all three allelochemicals had inhibitory effects on seedling disease incidence. High concentrations of biochanin A also inhibited the microbial activity. In contrast, water-soluble extracts stimulated microbial activity, and formononetin had no effect. But a counter-stimulation effect on microbial activities occurred when the concentration of water-soluble extracts was at 20 ng/g soil. I also found allelochemicals stimulated seedling leakage, providing chemoattractants for soil microbes. These results together highlight the important roles of allelochemical phytotoxicity in mediating soil microbial pathogenicity and seedling susceptibility to microbial attacks. Chemotactic microbes may take advantage of the allelochemical-induced damage.

Introduction

Soil microorganisms and allelochemicals from cover crop residues are both important biocontrol agents in sustainable weed management (Caesar, 2005; Charudattan, 2001; Liebman et al., 2000). The interactions between soil microbes and allelochemicals can greatly impact the overall weed suppressive potentials (Inderjit, 2005; Kaur et al., 2009). It has long been recognized that allelochemicals in the soil can influence soil microbial communities (Kong et al., 2008; Qu et al., 2008). However, the effects of allelochemicals on weed-antagonistic microbes are inconsistent in the literature. Both positive (A. Conklin, 1999; Mohler et al., 2012) and negative (Bhattacharya et al., 2010; Siqueira et al., 1991) effects of allelochemicals on microbial attacks on plants have been observed. These controversial outcomes hinder the utilizations of soil microbes and allelochemicals in weed management. Therefore, it is important to understand the specific roles of allelochemicals in influencing microbe-induced weed suppression.

Allelochemicals can negatively affect microbial activities. It is known that many allelochemicals produced by plants serve as defense chemicals against stress and pathogen attack (Dakora et al., 1996). The main phytotoxic compounds in red clover are isoflavones (Tsao et al., 2006), which have been identified as part of a broader class of anti-microbial molecules known as phytoalexins (Nicholson et al., 1992; Reynolds et al., 2003). My previous work (Chapter 2) found that the disease incidence on weed seedlings was lower in the early stage of residue decomposition, when the concentration of soil phenolics was high. It is possible that the dynamical changes of both allelochemicals and seedling disease over time caused a negative correlation between these two variables, but it is also possible that isoflavones compounds in red clover are adverse to weed-antagonistic microbes (Dakora et al., 1996; Nicholson et al., 1992).

Microbial weed suppression can also benefit from the allelochemicals from cover crop residues. For instance, the presence of microbes and residues offer higher weed suppression than either of them alone (A. E. Conklin et al., 2002; Mohler et al., 2012), and soil pathogen populations (Bonanomi et al., 2011; Rothrock et al., 1995) increase following residue incorporation in the soil. One possible reason for the synergistic interactions is that allelochemicals stimulate microbial attack by providing a carbon resource for microbes (Blum et al., 2000;Jilani et al., 2008). Another reason is that

allelochemicals can damage seedling cell membranes, disrupt membrane stability (Baziramakenga et al., 1995) and then induce seedling leakage (Z. Patrick et al., 1964; Toussoun et al., 1963). The injury induced by the allelochemicals may make the seedlings more vulnerable to microbial attack. Studies have found that pre-exposure of root lesions under aqueous extracts of timothy, rye, barley, broccoli, and broad bean could increase the prevalence of seedling disease (Z. A. Patrick et al., 1964; Toussoun et al., 1963). Moreover, because microbes demonstrate chemotactic movement towards seeds and seedlings (Begonia et al., 1994), the leakage induced by allelochemicals may provide cues to trigger pathogen movement towards germinating seedlings (Barbour et al., 1991; Broek et al., 1995). However, no clear evidence has shown this indirect pathway of stimulating microbial attack by allelochemicals. Many of the above examples involve well-known and culturable pathogens, but given the tremendous diversity of soil microbial communities, unknown microbes may also respond to the allelochemicals derived from cover crop residues. Examination of the whole soil microbial community by a modern sequencing approach can broaden the search scope of microbes that can participate in the chemotactic response.

In order to determine whether allelochemicals directly suppress microbial infection potential, in this study I manipulated the concentrations of allelochemicals in the soil. The first aim is to examine the effects of aqueous extracts of residues and two isoflavones on seedling infection and microbial activity. The second aim is to examine the effects of allelochemicals on seedlings exudates. Finally, I used the seedling exudates to determine the microbial chemotactic behavior, and I also identified these microbes by sequencing. I hypothesize that high a concentration of allelochemicals can inhibit seedling disease. If allelochemicals from cover crop residues are mostly utilized by seedling-associated microorganisms as a nutrient resource, then the microbial activity will increase. If the anti-microbial feature of allelochemical is more important, then the microbial activity will decrease. I also hypothesize seedling exudate can be chemoattractants for some soil microbes.

Methods and materials

Experiment 1: effects of allelochemicals on seedling disease

Preparation of residue extract

I planted red clover plants in the greenhouse of University of Illinois and harvested them at the bud stage after 14 weeks of growth. Freeze drying has been shown to be the best way to preserve the original forms of isoflavones in red clover as compared to oven drying (Tsao et al., 2006), so I freeze-dried the fresh plants to facilitate the extraction of water-soluble allelochemicals. I cut 20 g of the freeze-dried plants into 5-cm pieces and shook them in 400 ml sterilized, deionized water for 16 h at 23 ℃. I used cheesecloth to filter the plant residue, and then I centrifuged (4000g, 10 min) the liquid fraction to further remove particulate matter. I concentrated the liquid fraction five-fold by freeze-drying to a final volume of 80 ml. I stored the concentrated extract at -20 ℃ for use in the bioassays described below.

I collected field soil for bioassays in early May 2015, to a depth of 10 cm from an area at the Maxwell Trust site of the Crop Sciences Research and Education Center, Urbana, IL. The field plot had been maintained in a corn-soybean rotation for over 20 years. The soil at the field was a Catlin silt loam (*Oxyaquic Argiudoll*) with the following characteristics: 7% sand, 68% silt, 25% clay, 4.2% soil organic carbon, pH 7.2. I sieved the soil through a 2 mm sieve and stored in sealed plastic bags at 4 C for up to 1 week before use.

Experimental design

To examine the effects of allelochemicals on seedling infection, I conducted seedling growth bioassays using soil amended with a range of concentration of allelochemicals. The three allelochemicals were whole red clover extracts and two major isoflavones (Biochanin A and Formononetin) found in red clover. Biochanin A and formononetin counted as 70- 80 percent of the total isoflavones in the soil amended with red clover residues (Chapter 2). Thus, these two compounds should represent the behaviors of isoflavones in red clover.

In previous studies (Chapter 1 and 2), incorporation of 2% dry red clover into soil can give at most 28 ng phenolics per gram of soil. I wanted to bracket this concentration,

so my experiment used 0, 10, 20, 30, and 40 ng/g soil. I firstly did a test to know how much whole red clover extracts I needed to add to get a soil phenolic content comparable to previous studies. I empirically determined that adding 5 ml of whole red clover extracts to 10 gram of soil resulted in a measurable (by Folin-Ciocalteu's method; see Chapter 2 for details) soil concentration of 640 ng/g soil. Thus, to obtain 40 ng/g of soil in an 80 g soil mesocosm, I would need to add [40 ng/g soil * 80 g soil] / 640 ng/ml = 5 ml of whole red clover extracts. For all other target concentrations (e.g. 30 ng/g, 20 ng/g, etc.), I diluted the stored red clover aqueous extracts into proper concentration and added 5ml diluted extracts to all the mesocosm. For the other two isoflavones (biochanin A and formononetin), I used reagent-grade chemicals to prepare the solutions. Chemicals were purchased from Sigma-Aldrich. The soil phenolic content without allelochemical addition was under detection, so I added 5ml distilled water as 0 ng/g soil. The total number of mesocosms was (3 chemical treatments x 4 levels of concentration + water control) x 6 replications = 78.

Bioassays of Seedling disease incidence

I used the seed germination bioassay technique of Dabney and colleagues (1996) to assess the allelochemical effects on weed seedling disease incidence with the following modifications. I used IdaGold mustard (*Sinapis alba*) as the target weed because it is a common weed of temperate agroecosystems. I germinated seeds on a petri dish two days before the bioassay. I selected seedlings of about 20mm length to construct the bioassay.

Each bioassay unit was constructed from a different, single mesocosm. I placed 10 mustard seedlings in a line 10 cm from the top edge a double layer of 25 cm by 38 cm germination paper (Anchor Paper, St. Paul, MN) moistened with 20 ml of sterilized, deionized water. Then I spread the 80 g of soil from a mesocosm in a 12cm wide band, about 6 cm from the top edge of germination paper, to cover the line of seedlings. I placed another moistened sheet of germination paper on top of the seedlings and soil, and rolled this entire assembly from the short edge to create a cylinder. I wrapped and sealed each cylinder in a plastic Zip-lock bag to maintain soil moisture content. I incubated these bioassay units with seedlings oriented “up” in the upright cylinder in a Conviron 125-L incubator (Controlled Environments Limited, Manitoba, Canada) for 7 days with a 16 h light:8 h dark cycle (25 ℃ and 20 ℃, respectively).

After 7 days of incubation, I deconstructed each bioassay unit. I recorded the length of visible necrotic lesions on the seedlings, which I considered to be disease incidence for the purposes of this study.

Statistical Analysis

I performed linear regression and ANCOVA to determine the effects of allelochemicals on the length of diseased seedlings, and whether the effects were different for different allelochemicals.

Experiment 2: allelochemical effects on the activities of seedling attached microbes

The aim of this experiment is to determine whether the allelochemicals can stimulate or inhibit microbial activities. Active plant pathogens are most likely to be found in microbial communities that are closely associated with infected seedlings, so I collected diseased seedlings to assess the responses of seedling attached microbial communities to the allelochemicals and whole red clover extracts. After I recorded diseased seedlings of one bioassay, I carefully removed soil that was adhering to the seedlings and put all the diseased seedlings into one 15ml centrifuge tube. I added 2ml distilled water into each tube to wash off microbes attached to the seedling by shaking the tube gently for 10min. To control the seedling to seedling variability, I split each microbial suspension into two parts that I assayed separately: one part was added with the allelochemicals, and the other part was the control that was just added with water. I measured microbial respiration as a proxy for microbial activity. I used MicroResp™ to assess microbial respiration. MicroResp™ is a colorimetric method based on the color change of a pH indicator dye caused by the release of CO_2 by microbial communities (Chapman et al., 2007). I added 500ul of the microbial suspension to a deep-well microplate (1.2 mL capacity, 96-deep-well microplate, NUNC) with the chemicals. Microbes exposed to a certain allelochemical level in experiment 1 received the same allelochemical level in this experiment, e.g., I added 40 ng biochanin A in the cell which contained microbial suspension from bioassay treated with 40 ng/g soil phenolics in experiment 1. The second microplate contained 150 μL detection gel per well. The detection gel was made by cresol red dye (12.5 ppm), potassium chloride (150 mM) and sodium bicarbonate (2.5 mM) set in a 1% gel of noble agar. I measured the indicator dye

at 590 nm (spectrophotometer) immediately before I sealed it to the deep-well plate. These two plates were sealed together with a silicone seal, with connecting holes between the corresponding cells. I incubated the assembly at room temperature for 6 hours. During this time, microbial respiration in each soil well will produce a proportional color change in the corresponding indicator well. I measured the indicator dye again after incubation.

Statistical analyses

For this experiment, I used a paired experiment to control the sample to sample variance. Thus, for each pair, I firstly standardized the microbial respiration by subtracting respiration in the water control from respiration in the allelochemical sample. I used a t-test to determine whether the allelochemicals significantly changed the microbial respiration. Because no linear relationship was observed from the responses of respiration to allelochemical concentrations, I used ANOVA test the main effects of allelochemical, concentration levels and their interaction on respiration.

Experiment 3: composition of carbon metabolism in seedling exudate

Most previous studies on seedling leakage used a pure chemical solution to pre-treat seedlings (Z. A. Patrick et al., 1964; Toussoun et al., 1963). But using a pure chemical in a petri dish often exaggerates the potential toxic effect of the allelochemical compared to the effect in the soil (Inderjit et al., 2008; Kaur et al., 2009). To better reflect the real field condition, I exposed mustard seedlings to soil mixed with allelochemicals. Because microbial communities and allelochemicals in soil can both influence seedling exudates, I used a 0.2-micron filter (EMD Millipore Corp, Fisher Scientific) to block the soil microbial community from directly accessing the seedling, while still allowing chemical exchange with soil. As in experiment 2, I germinated wild mustard seeds two days before the experiment. On the day of the experiment, I mixed fresh soil with the proper amount of allelochemical solution (red clover residue extract, biochanin A or formononetin) to get a range of target concentrations the same as experiment 1, which were 40 ng/g soil, 30 ng/g soil, 20 ng/g soil and 10 ng/g soil. The control was soil mixed with distilled water. Each treatment had 4 replications. I put three 20-mm seedlings in the middle of a petri dish and covered them by a piece of filter. The filter was sealed by tape

and then covered by 20g allelochemical mixed soil. I allowed the seedlings to be exposed to the allelochemicals for 16 hours. I used 2ml methanol: water (95:5) to wash the seedlings and collected seedling washes for following metabolite compound analyses.

Metabolite Derivatization, gas chromatography and mas spectrometry (GC/MS)

I used GC/MS to characterize the compositions of metabolites in whole red clover extracts and seedling exudates. The GC/MS procedure was run in batches, as the lifetime of derivatized samples is only about 48 hours at room temperature. Sample extraction was also done in a batch mode. I have established a protocol in which batches had almost no deviation between runs (less than 0.8%). Dried extracts were derivatized with 80 µl methoxyamine hydrochloride (Aldrich, USA) (40 mg ml^{-1} in pyridine) for 60 min at 50^0C and then with 100 µl MSTFA+1% TMCS (Thermo Sci., USA) at 70^0C for 120 min and followed by 2-hour incubation at room temperature. Five microliters (5 µL) of the internal standard (hentriacontanoic acid (10 mg ml^{-1}); Sigma, USA) were added to each sample prior to derivatization. Samples were analyzed on a GC/MS system (Agilent Inc, Palo Alto, CA, USA) consisting of an Agilent 7890 gas chromatograph, an Agilent 5975 mass selective detector, and a HP 7683B autosampler.

Gas chromatography was performed on a ZB-5MS (60 m × 0.32 mm I.D. and 0.25 µm film thickness) capillary column (Phenomenex, CA, USA). The inlet and MS interface temperatures were 250 C, and the ion source temperature was adjusted to 230 C. An aliquot of 1 µL was injected with the split ratio of 10:1. The helium carrier gas was kept at a constant flow rate of 2 ml min^{-1}. The temperature program was: 5-min isothermal heating at 70 C, followed by an oven temperature increase of 5 C min^{-1} to 310 C and a final 10 min at 310 C. The mass spectrometer was operated in positive electron impact mode (EI) at 69.9 eV ionization energy in m/z 30-800 scan range.

Statistical Analysis

I performed linear regression and ANCOVA to determine whether the total concentrations of seedling exudates increased as seedling were exposed to high concentrations of allelochemicals, and whether the effects were different for whole red clover extracts, biochanin A and formononetin. I used Permutational multivariate

ANOVA to test the effects of allelochemical and concentration on the composition of metabolites in seedling exudates.

Experiment 4: Responses of soil microbes to seedling exudates

Chemotaxis experiment

To determine whether some soil microbes can actively move toward seedling exudates, the occurrence of chemotaxis was studied. First, I used the highest concentration of whole red clover extracts to treat seedlings. The process was the same in experiment 3. After the treatment, I used 1 ml distilled water to wash one seedling. I concentrated the water-soluble exudates to half of the initial volume by vacuum centrifuging at room temperature. I filter-sterilized exudates through a 0.22-micron filter and then stored them at -20^{o}C until further use.

To get soil microbial suspension, I shook 5g fresh soil in 15 ml Phosphate buffered saline (PBS) buffer for 12 hours. I centrifuged the soil-PBS mixture for 5 min at room temperature and then collected the water phase. The water phase was filtered through Whatman® qualitative filter paper (Grade 4) to remove solid particles. The water phase was used immediately in the following chemotaxis experiment.

For the chemotaxis experiment (modification of Klerks, 2007), I filled micro-capillaries (volume of 50 ml, diameter of 1 mm) with 0.2% of Hoagland's agar, including 0.5% of the metabolism marker 2,3,5-triphenyl tetrazolium chloride to indicate microbial movement. I horizontally positioned one end of a capillary inside a 0.2 ml tube (also horizontal and sealed with parafilm) to allow contact with the microbial suspension or control solution. On the other end of a capillary, I placed another 0.2 ml tube containing the seedling exudate sample or negative control (water, Hoagland's solution or PBS buffer). I carefully wrapped the junction area with parafilm to prevent evaporation. Each treatment had eight replications. I incubated the capillaries horizontally two days at 37 C prior to the observation of chemotaxis by color transition inside the capillaries.

To characterize microbes that moved into the capillaries, after observing the color transition, I collected the solutions inside capillaries and the microbial suspension separately into new tubes. Samples were stored in -80 C before the following processes.

Illumina sequencing

Because I didn't observe PCR products from fungal PCR, I only analyzed bacterial communities. The bacterial communities were accessed by sequencing the V3 – V4 region of 16S rDNA using the PCR primers 515F and 926R (Caporaso et al., 2011; Lane, 1991). The fungal communities were accessed by sequencing the ITS2 region of ITS using the PCR primers ITS3 and ITS4 (White et al., 1990).

Sequence pre-processing

I firstly merged pair-ends sequence reads by FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). I filtered merged sequences by following requirements: average quality score larger than 30, and 95% percent of bases have quality score larger than 30. Then I used USEARCH version 8.0.1517 (Edgar, 2010) to do the following processes. I sorted sequences by abundance and then removed singletons. I clustered sequences at 97% similarity within USEARCH (UCLUST) to select representative sequences for each OTU. I used USEARCH/UCHIME to detect chimeras by comparing to Gold database (Kyrpides, 1999). The representative sequences of bacteria were aligned and assigned taxonomic information by Greengenes database within QIIME (DeSantis et al., 2006).

Statistical Analysis

I standardized microbial data by Hellinger transformation (Legendre et al., 2001). I performed Permutational multivariate ANOVA to determine whether different substrates attracted different microbes. To identify which microbes that moved specifically to seedling exudates, I removed microbial OTUs that were found in any control treatments from the dataset of seedling exudate treatment.

Results

Bioassay of seedling disease incidence

Increasing concentrations of allelochemicals significantly reduced the percentage of diseased seedlings (ANCOVA, P-value<0.001) (Figure 5.1), and the overall effect did not differ among three allelochemical treatments (ANCOVA, P-value = 0.12). However, the slope of the regression for biochanin A was significantly more negative than the other

two (coefficient = -0.01, P-value <0.01), while formononetin and residue extract had similar negative slopes (coefficient= -0.006, P-value <0.01).

Effects of allelochemicals on the respiration of seedling attached microbes

For the microbial communities associated with infected seedlings, there were significant main and interaction effects (P-value <0.05) of allelochemical and concentration levels on their activity as assessed by respiration rates. Aqueous extracts of residues significantly increased (P-value <0.05) the microbial respiration, with the greatest increase apparent at the lowest aqueous extract concentrations. In contrast, biochanin A decreased respiration but only significantly in high concentration: 30ng and 40ng. Formononetin didn't show any significant effect on respiration.

Composition of metabolites in seedling exudates

In total, 188 metabolites belonging to 12 chemical classes were detected from seedling exudates. The total concentration of metabolites increased as seedlings were exposed to higher concentration of allelochemicals (Figure 5.3) (coefficients of linear regression: whole red clover extracts: 1603.8, biochanin A: 1046,9, formononetin: 1068.6, P-value <0.005 for all three treatments), but the effects of allelochemicals on seedling exudates were not significantly different among treatments (ANCOVA, P-value=0.14). The composition of metabolites was significantly different among the chemical concentrations (R^2=0.29, P-value<0.05), but not different among allelochemical treatments (R^2=0.066, P-value=0.069).

The seedling exudates of wild mustard resulted in metabolic activity (red coloring) inside the microcapillaries (4 out of 8 positive) (Figure D.1). Each control tube was negative for the color transition that would indicate microbial respiration inside the capillary tube (24 out of 24). A chi-square test on the total number of positive samples (red coloring) between the control tubes and the tubes connecting to seedling exudates showed a significant difference (Chi-square = 9.5238, P-value=0.002).

I detected a total of 1690 bacterial OTUs from all the treatments. Significantly different microbial communities were found between the controls and the seedling exudate treatment (Permutation ANOVA, P-value<0.001), and marginal significant differences were detected between the microbial source pool (microbial suspension) and

the seedling exudate treatment (Permutation ANOVA, P-value=0.03). Only 281 OTUs were detected in seedling exudate treatment and none of the controls. For these bacteria that responded uniquely to exudates, *Proteobacteria* was the dominant phylum. At the level of class, the top five abundant classes are *Gammaproteobacteria* (23.7%), *Bacilli* (17.4%), *Sphingobacteria* (13%), *Deltaproteobacteria* (12.3%), and *Planctomycea* (8.5%) (Figure 5.4). At the order level, the top three orders were *Legionellales* (21%), *Bacillales* (17.4%), *Sphingobacteriales* (8%). 28 OTUs were founded in more than half of the replicates. Five OTUs were identified at the order level as *Legionellales.* Four OTUs were *Sphingobacteriales* and four OTUs were *Desulfuromonadales* (Table 5.1).

Discussion

My results support the hypothesis that allelochemicals in red clover residues have mixed effects on microbial weed antagonists. High concentrations of allelochemicals inhibited seedling disease incidence (Figure 5.1) and microbial respiration (Figure 5.2). But allelochemicals could also stimulate seedling exudates (Figure 5.3), providing chemoattractants for weed antagonistic microbes (Figures 5.4 and D.1). These results suggest that besides the well-known direct allelopathic effects on weeds, allelochemicals from green residues can also indirectly influence weed performance by mediating seedling exudation and soil-borne weed antagonistic microbes.

Previous works showed inconsistent results about the effects of incorporation of crop residues on disease incidences on plants. Stimulation (Bonanomi et al., 2011; Rothrock et al., 1995) and reduction (Blok et al., 2000; Hoitink et al., 1999) of soil pathogenicity were both observed. The inconsistence may be due to the different environmental conditions and experiment approaches. My results agree with the previous studies showing a reduction of seeding disease (Figure 5.1). The general mechanism proposed by previous studies is that the rich nutrients in crop residue enhance microbial growth and competition against specific soil pathogens (Bailey et al., 2003; Mazzola, 2004). However, my results suggest a different mechanism. The reduction of plant disease may be the result of the inhibitory effects of residue-derived chemicals on soil pathogens (Figure 5.2).

Although all three allelochemicals showed inhibition of seedling disease incidence (Figure 5.1), only biochanin A showed inhibition of the respiration from diseased seedling-associated microbes. Water-soluble red clover extracts stimulated respiration, and formononetin had no effect (Figure 5.2). Because the aqueous red clover extracts were rich in sugar substrates (Figure D.2), it is not surprising that microbial respiration was stimulated in these treatments (Figure 5.2). But a counter-stimulation effect on microbial respiration occurred when the concentration of aqueous extracts increased above 10 ng/g soil (Figure 5.2). The dose effects of biochanin A and aqueous red clover extracts were both nonlinear. Respiration dropped within a specific range of concentrations (10 - 20 ng/g soil for water extract, and 20 - 30 ng/g soil for biochanin A) and thereafter did not change as concentration increased. There may be a threshold point at which the respiration changes abruptly from one phase to another. Aqueous red clover extracts had a lower threshold than biochanin A, suggesting that a mixture of different allelochemicals generated higher inhibition than a single chemical. This threshold relationship also indicates the microbial community may have a "resistance" to a range of allelochemical inputs. Experiments with a higher resolution in this threshold range of concentration may better establish the thresholds for microbial responses. Formononetin showed no effect on microbial respiration. One possible reason is that formononetin influenced microbial activity, but this was not reflected in the microbial respiration. A study found that other microbial characteristics, like biomass carbon and carbon utilization efficiency, can also indicate the toxicity of aqueous plant residue extracts (Qu et al., 2008). Examination of other microbial characteristics may reveal the effects of formononetin on microbes.

The amount of red clover residues (2% by weight) used in previous studies (Chapter 2 and 3) is comparable to the incorporation rate in field condition. Previous studies (Chapter 2 and 3) showed that the highest total phenolic concentration from this amount of residue addition was 25-30 ng/g soil, and the highest concentrations of biochanin A and formononetin were 20 ng/g. Because the inhibition of microbial respiration occurred here at a higher concentration than may be typical in green manured soil, the inhibition by biochanin A may be less likely under field conditions, unless the chemical was locally concentrated in the soil.

The microbial respiration presents the overall responses of whole community. However, in the soil community, some microbial groups may be inhibited by biochanin A and formononetin, and some groups may be stimulated (Furbo et al., 2011; Shaw et al., 2008). A lot of microbes metabolize sugars, but only a few groups of microbes can metabolize phenolic compounds as a sole carbon source (Blum et al., 2000; Ozan et al., 1997),

Several previous studies provide evidence that allelochemicals can make the seedling an easier target for microbial attack (Z. Patrick et al., 1964; Toussoun et al., 1963). Here I show for the first time a specific linkage between the allelochemical-induced leakage and microbial chemotaxis, supporting the notion that allelochemicals can assist microbial attack by inducing chemical cues for microbes. Phenolic compounds can impair root membrane functions, consequently causing iron leakage (Baziramakenga et al., 1995; Yu et al., 1997). I found a clear trend that higher allelochemical concentration induced more seedling exudates, but the compositions of metabolites in exudates were not different between three allelochemical treatments (Figure 5.3). This suggests that these they may all have similar functions in inducing seedling leakage.

Seedling exudates are important chemoattractants for soil microbes to initiate cross talk with plant roots (Broek et al., 1995; Turnbull et al., 2001). The majority of compounds in seedling exudates were sugar and organic acids (Figure 5.3), which are important attractions of the soil microbes (Bacilio-Jimenez et al., 2003; Begonia et al., 1994). The chemotactic responses of soil pathogens to seedling exudates may be critical for infection (Yao et al., 2006). For example, chemotaxis-deficient isolates of *Rhizobium* appear to be strongly disadvantaged in infecting plant roots comparing to chemotactic isolates (Malek, 1992). In my experiment, I found chemotactic OTUs in families that are known plant pathogens such as *Xanthomonadaceae.* Genome analysis supports their mobile abilities and chemotactic potentials (Pieretti et al., 2009). Moreover, because many opportunistic pathogens only express pathogenicity or virulence factors at a high cell density (Lugtenberg et al., 2009), chemotaxis might be not only important for opportunistic pathogens to compete with other soil bacteria in approaching seedling, but also important for them to reach the density for pathogenicity.

Three chemotactic OTUs discovered here were also found as putative weed-suppressive microbes in my previous studies (Chapter 3 and 4). They are OTUs in genus of *Aquicella, Alkanindiges* and *Pedobacter.* Their chemotactic abilities may help them target seedlings. Because of the inhibitive effects of allelochemicals on microbial pathogenic activities (Figure 5.1 and 5.2), whether the chemotactic microbes can take advantage of the chemical induced seedling damage also depends on whether they can stay active under the phytotoxic pressure. The highest concentration of isoflavones (40ng/g soil) still could not completely inhibit disease incidence and microbial respiration (Figure 5.1 and 5.2). In Chapter 3, I found that the abundance of some soil microbes were not influenced or even enriched by the additions of red clover residues. These microbes may be resistant to the anti-microbial effects of isoflavones (Morrissey et al., 1999; Pedras et al., 2005). For the three weed suppressive microbial OTUs with chemotactic potentials, none of them showed significant changes with the additions of red clover residues in previous studies (Chapter 3 and Chapter 4). They seems at least not be inhibited by allelochemicals. In cover crop agroecosystem, it would be best that the biocontrol microbes can be stimulated by cover crop residues, but it would be also desirable that the biocontrol microbes are not toxified by phytotoxic chemicals. Right now we had little knowledge about their interactions with plants in literatures, and more work is needed to understand the basic ecology of these organisms and how to use them to our advantage.

Conclusion

Successful biological control of weed growth depends on several factors for soil microbial communities to function in the soil and the rhizosphere (Charudattan, 2001; Kremer et al., 1996). This study is the first dealing with the mixed effects of allelochemicals on weed antagonistic microbes. I illustrate the important roles of allelochemical toxicity in mediating the microbial pathogenicity and seedling susceptibility. Allelochemicals can not only damage weed seedlings to make them more vulnerable to attack from the pathogens in rhizosphere, but they also induce seedling exudates to attract chemotactic pathogens from the bulk soil. Because microbes with potentials in chemotaxis and allelochemical-stimulation may take better advantage of the

allelochemical-induced seedling damage than allelochemical-inhibited microbes, these two traits may be especially important for the selection of weed biocontrol microbes for a green manure system.

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Tables and figures

Table 5.1. Chemotactic bacterial OTUs that were detected in at least half of replications (4 of 8 replications).

Order	Number of OTUs
Legionellales	5
Desulfuromonadales	4
Sphingobacteriales	4
Gemmatales	2
Pseudomonadales	2
Rhodocyclales	2
Acidobacteriales	1
Chromatiales	1
Clostridiales	1
Dehalococcoidales	1
Desulfovibrionales	1
Planctomycetales	1
Rhizobiales	1
Syntrophobacterales	1
Xanthomonadales	1
Total	28

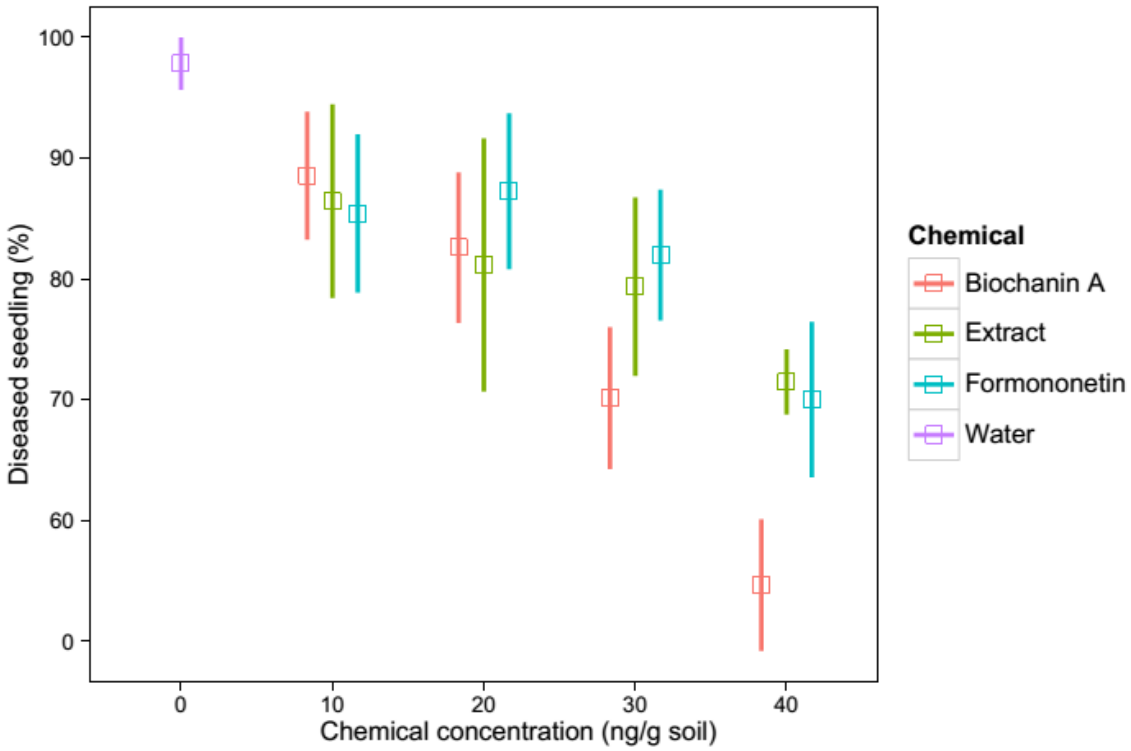

Figure 5.1. Percentage of diseased mustard seedlings in bioassays with different allelochemical concentrations. Concentrations of aqueous red clover extracts are expressed as the target concentration of soil phenolics, not the total extract concentration. Error bars are standard errors from six replicate analyses.

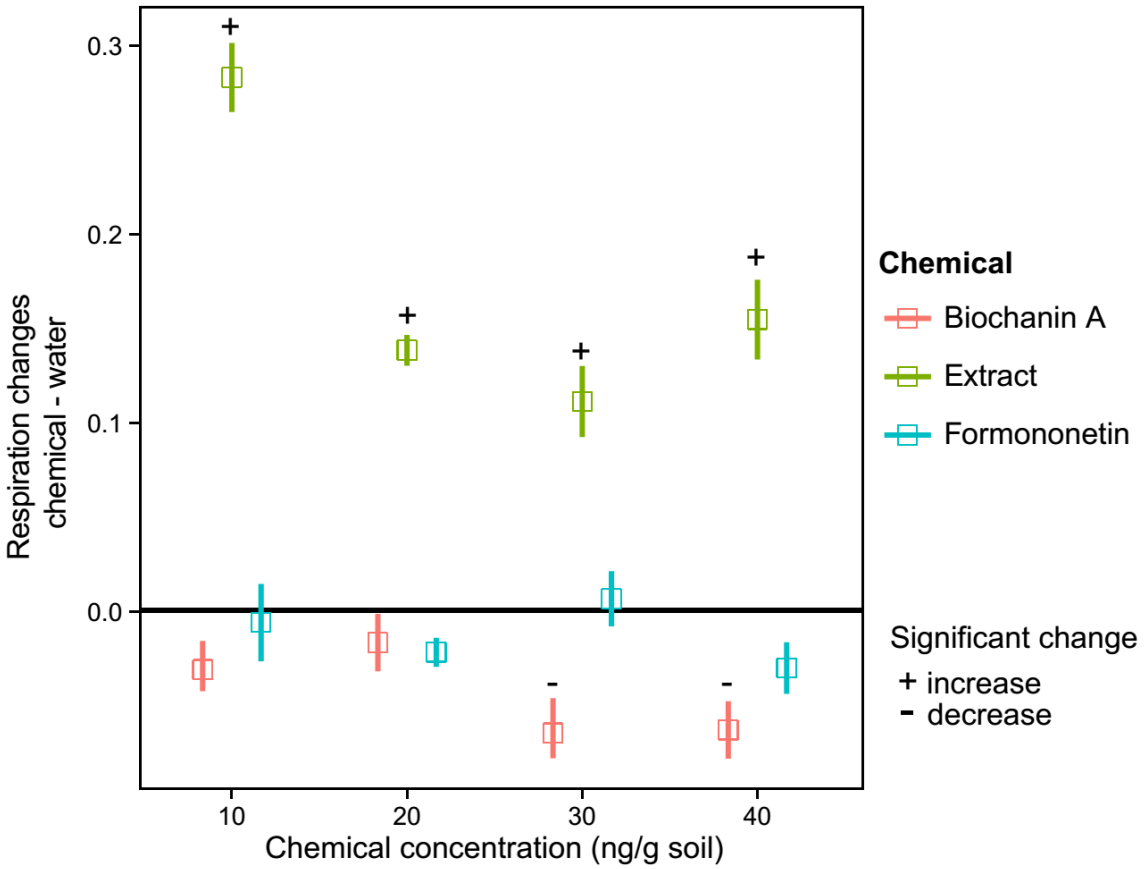

Figure 5.2. Respiration of microbial communities attached to the infected seedlings in different allelochemical concentrations. Concentrations of aqueous red clover extracts are expressed as the target concentration of soil phenolics, not the total extract concentration. Error bars are standard errors from six replicate analyses.

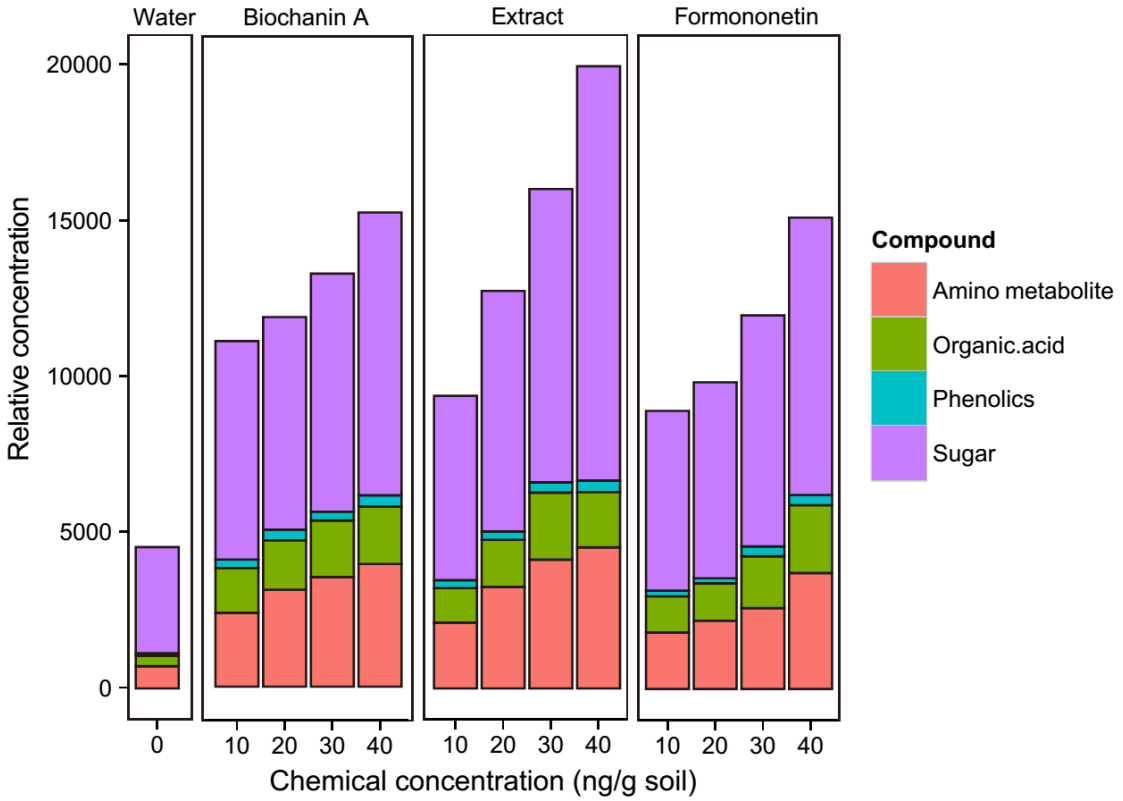

Figure 5.3. Relative concentrations of major metabolites from seedling exudates. Concentrations of aqueous red clover extracts are expressed as the target concentration of soil phenolics, not the total extract concentration.

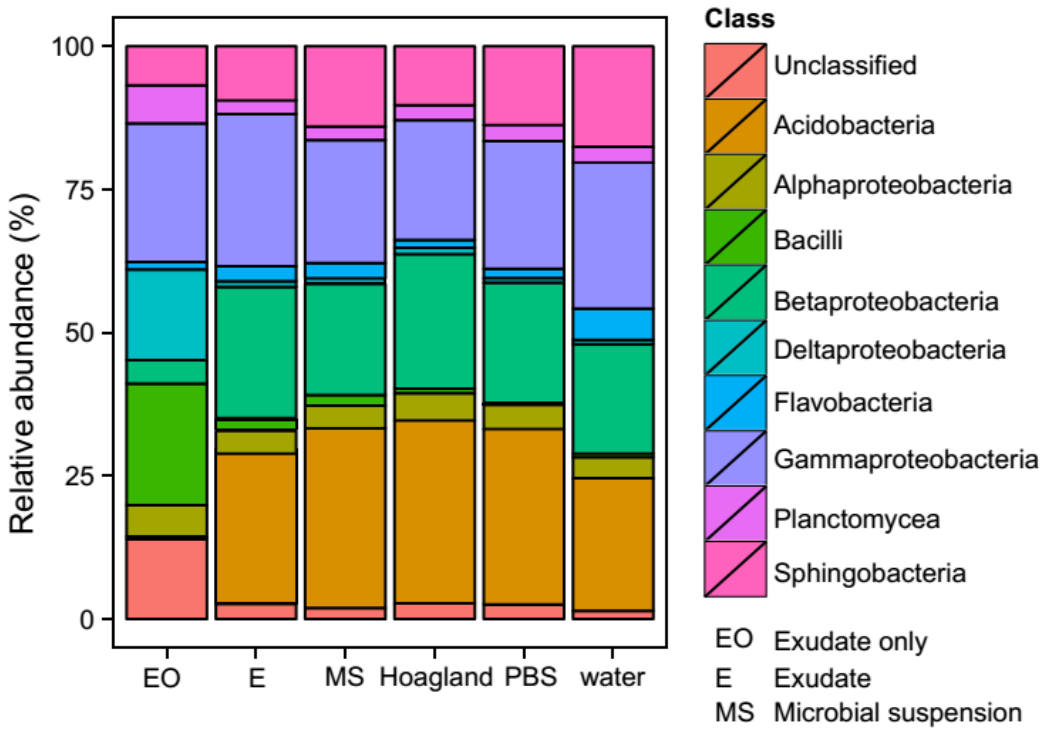

Figure 5.4. The relative abundance of the top 10 bacterial classes in capillary tubes that were "baited" with seedling exudates and various controls (Hoagland solution, water, PBS).Also shown are the top 10 OTUs in the "source" microbial suspension. Exudate only indicates bacterial OTUs that responded to exudate bait and none of the controls.

CHAPTER 6: CONCLUSIONS

Summary of Findings

In this dissertation, I applied a combination of greenhouse and molecular approaches to the study of the roles of allelochemical and microbes in weed suppression. I explored the interactions between cover crop residues and microbial communities across a variety of scales.

The interaction between cover crop-derived and microbial-derived weed suppression was negative. This negative microbe-by-residue interaction is likely to be the result of a rapid microbial degradation of the allelochemicals in cover crop residues (Inderjit, 2005; Kaur et al., 2009). As shown in Chapter 2, the phenolic compounds were lower in the presence of live microbes than in sterilized soils. It is also possible that the residue-associated allelochemicals negatively affected the weed-suppressive microbes. I found that the seedling disease incidence was low in soil with high phenolic contents (Chapter 2 and Chapter 4). In Chapter 5, I directly manipulated the concentrations of allelochemicals in the soil. Results confirmed the inhibitive effects of allelochemicals on disease incidence.

An important piece of this dissertation is the identification of a subset microbes with putative weed-suppressive activities from the entire microbial communities. I proposed a trait-based method to identify putative weed suppressive microbes in Chapter 3. A similar concept was used in Chapter 4. The putative weed suppressive microbes discovered in Chapter 3 and Chapter 4 consist of a diverse group of microbes including known pathogens and saprophytes, and unknown microbes. These microbial taxa include well-known plant pathogens, such as *Fusarium* and *Pseudomonas*. The most abundant disease-promoting fungal taxa found in Chapter 3 is *Myrothecium verrucaria*, which has been developed as biocontrol agent to suppressive kudzu (*Pueraria lobata*) (Boyette et al., 2002). Some of the microbial taxa were repeatedly found in the two chapters. Given the large variation of microbial communities across these two studies, the consistent observations of these microbes suggest that they are widespread in different types of soil, they consistently play important roles in the weed suppression, and they have positive responses to the addition of residues. Although my identification of putative weed-

suppressive microbes was entirely based on correlations, I believe that my results can provide valuable information to future studies on selecting biocontrol agents. I also believe that the trait-based method will provide a conceptual criteria for weed ecologists to discover novel microorganisms or populations.

A diversity of biocontrol agents may reside in the soil, but they need special management considerations to enhance their activities. This dissertation shows that microorganisms respond to management practices, so the farmers can use these management practices to indirectly manipulate soil microorganisms. My results also suggest that the weed suppressive microorganisms respond to management practices at different scales. Long term-scale management, such as tillage and organic management, affected the composition of weed-suppressive microbial communities. As shown in Chapter 4, soil microbes in the organic system had higher weed suppression potential than in the no-tillage system. The short term-scale management, such as the incorporation of cover crop residues, also impacted the weed-suppressive microbes (Chapter 2 and Chapter 4). On the small scale, cover crop residues stimulated some weed-suppressive microbial taxa (Chapter 3 and Chapter 4). Cover crop residue-associated allelochemicals damaged weed seedlings and induced seedling leakage, which may stimulate attacks from chemotactic pathogens (Chapter 5). These observations suggest that it is possible to have a specific synergistic interaction under the overall negative microbe-by-residue interaction, since the drivers and responses are happening at different scales. These three scales of response should be considered in developing weed management strategies. Management practices, which can encourage weed-suppressive microbes on all three scales, may improve biological weed control.

Future directions

Although I carefully set up the red clover residue treatments to mimic the incorporation practice in the field, the findings are still based on greenhouse-based bioassays. The effects observed from well-controlled greenhouse experiments may diminish under field conditions (Barnes et al., 1987; Nilsson, 1994). This means, the suppressive effects observed in this study may not exactly represent actual weed-suppressive effect in the field. Future studies will be necessary to replicate these results

under field conditions, and to test the effect size of weed suppression from red clover residues.

This study demonstrates the effects of agricultural management practices on the microbial interactions with cover crop residues and their associated weed-suppressive potentials. However, this study provides a snapshot rather than a longitudinal study. As the temporal dynamics is important for understanding the ecological process (Walker et al., 2010), more studies on the long-term effects of management practices are needed, which can be a multiple-year study that keeps tracking the changes of weed suppression or a chronosequence study that has fields with different management age. Those studies may test if there is a linear relationship between the management age and the weed suppression, and show if there are multiple potential trajectories for the succession of microbial communities.

This dissertation considers the agricultural management as a "system level effect", thus, it remains to determine the environmental factors that play a strong role in shaping the composition and functions of microbial communities. One possibility is that the diversity of organic substrates incorporated in the soil changes the soil microbial community composition. For example, I found that the microbial communities in the organic system had a higher diversity and more correlations between individual taxa than that in the no-tillage system. The high diversity of microbial community may be correlated with the high diversity of substrate inputs in organic farms. To further elucidate the importance of this factor, a study may first survey a large number of sites with different diversity of organic substrates; then, manipulate environmental conditions to test the relationship between the diversity of organic substrates and microbial composition and function.

Sequencing techniques provide detailed insights on microbial community, which make it possible to describe plant-microbial interaction a more analytical way. This dissertation provides important baseline data regarding the putative weed-suppressive microbes, although more work still needs to be done. A major question is how to distinguish pathogens from saprobes during the process of microbial seed and seedling attack. In other words, which microbes are the initiators of pathogenic attack on the seed coat, and which microbes are following saprobes that are attracted by the exposed interior

carbon resource? A study using DNA-based stable isotope probing (SIP) may be useful to identify the active initiators from saprobes. We may be able to only label the interior of seed by incubating plant with ^{13}C during the seed filling period. If we can obtain seeds that only have ^{13}C inside but not in the coat, then we can distinguish the initiators without ^{13}C from followers with ^{13}C. Another major unknown is the actual function of the putatively weed suppressive microbial taxa. I speculate the ecological roles of the detected taxa based on what has been previously described in other systems. Genome analyses of these microbes may provide evidence of their potentials in infecting plants, killing plants or chemotaxis (Allen et al., 2005; Riesenfeld et al., 2004). However, to confirm the weed-suppressive effects, we need to cultivate these microbes and then inoculate them back to soil (Charudattan et al., 2000). In the long term, the capability of cultivate individual microbial taxa may be improved. I may be able to confirm the functions of these microbes.

Conclusions

In summary, this dissertation contributes to the weed biocontrol in green manure agricultural systems. Traditional weed biocontrol approach relies on the application of microbial biocontrol agents into the soil. However, it is challenging to assure the efficacy of these microbes and minimize their spillover to crops (Charudattan et al., 2000). This study suggests soil-borne microbial communities are high-potential but untapped sources of weed suppression. Thus the promotion of natural borne soil communities could be an alternative approach to the application of biocontrol organisms. Many potential weed-suppressive microbes may exist in the soil, but special management strategies need to be considered to enhance their activities (Charudattan et al., 2000; Kennedy et al., 1996). This study found that incorporation of fresh cover crop residue not only provides allelochemical weed suppression but can be a tool to stimulate some naturally occurring weed-suppressive microbes. I elucidated the microbe-residue interaction and identified putative weed-suppressive microbes from the super complex and diverse soil microbial communities. However, this is only the first step in using ecological theories to advance agricultural weed biocontrol methods. It is envisaged that studies on putative weed-suppressive microbes will improve weed management.

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APPENDIX A: SUPPLEMENTARY INFORMATION FOR CHAPTER 2

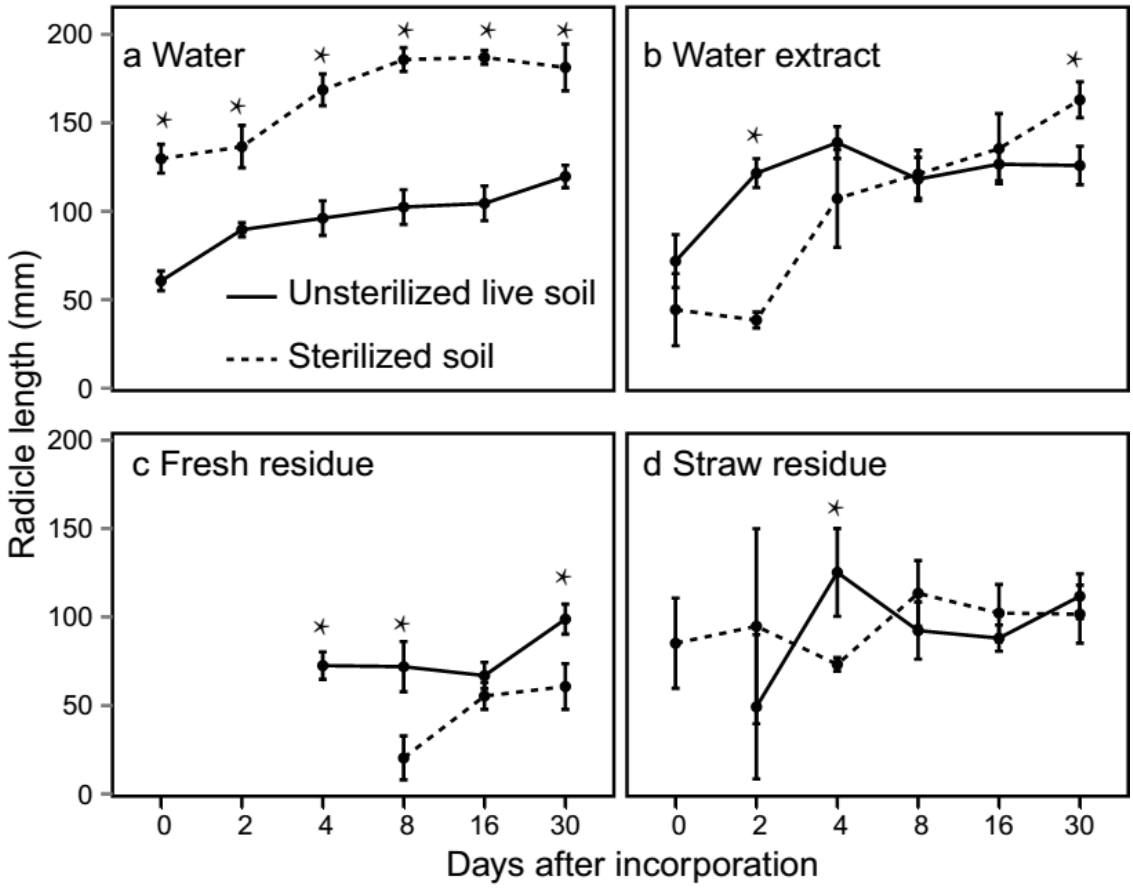

Figure A.1. Inhibition of radicle elongation by red clover residues and soil microorganisms varies over time. Seedling radicle length of mustard in sterilized and live soil is shown for treatments exposed to (a) water, (b) water-soluble extracts, (c) fresh residues, and (d) straw residues. Error bars are standard errors from ten replicate analyses. Stars indicate comparisons that were determined to be significantly different at alpha = 0.05 by a Tukey's HSD test.

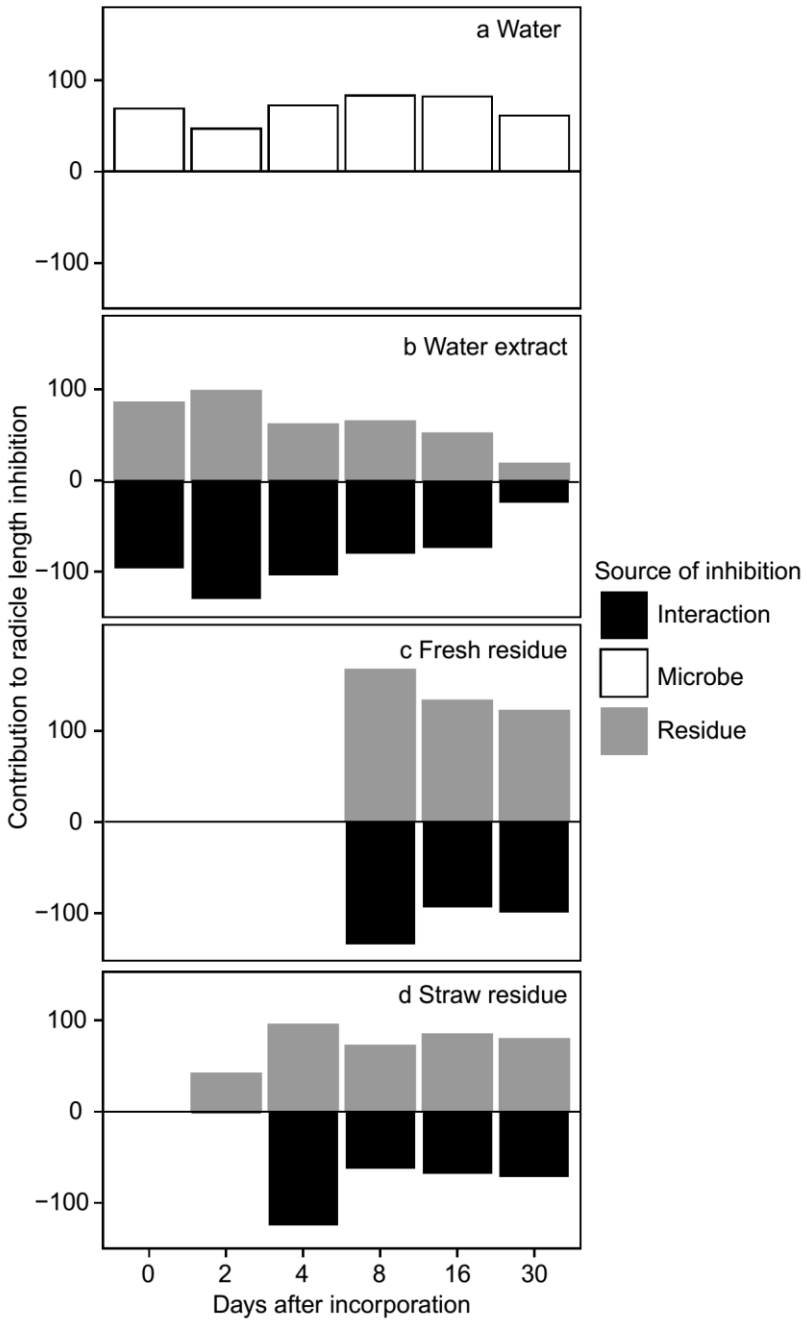

Figure. A.2. Antagonistic interactions between soil microorganisms and red clover residues differ between residue fractions. Bars indicate the strength of microbe-only, residue-only, and microbe-by-residue contributions to radicle elongation inhibition.

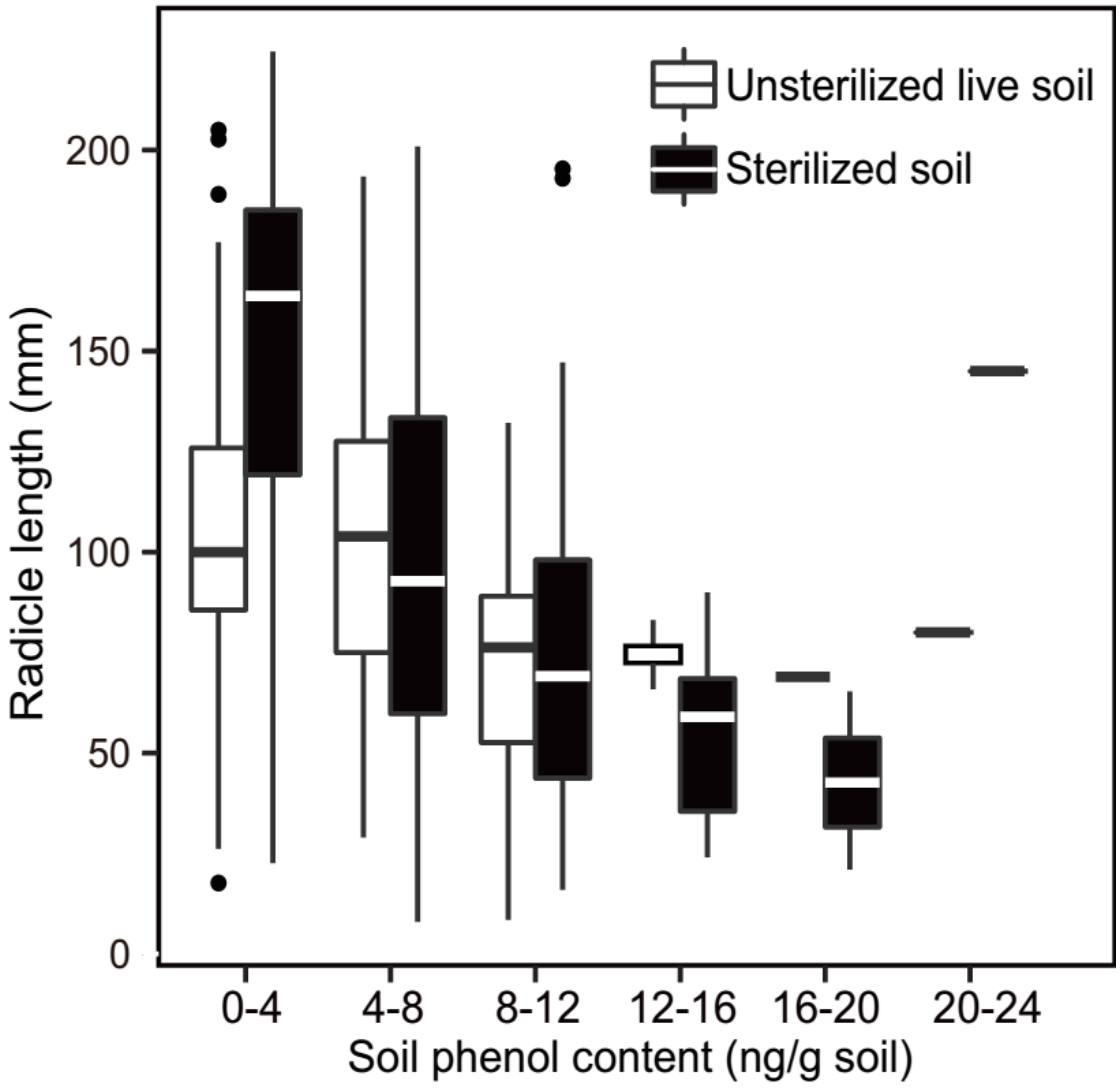

Figure A.3. Radicle elongation is inhibited by high concentrations of soil phenols.

APPENDIX B: SUPPLEMENTARY INFORMATION FOR CHAPTER 3

Table B.1. The effects of environmental factors on total soil microbial communities by Permutational ANOVA.

		R^2	P-value
Bacteria	Soil fraction (soil and seedling)	0.44573	0.001
	Residue fraction	0.02897	0.001
	Day	0.01579	0.001
Fungi	Soil fraction (soil and seedling)	0.14955	0.001
	Residue fraction	0.08866	0.001
	Day	0.04031	0.001

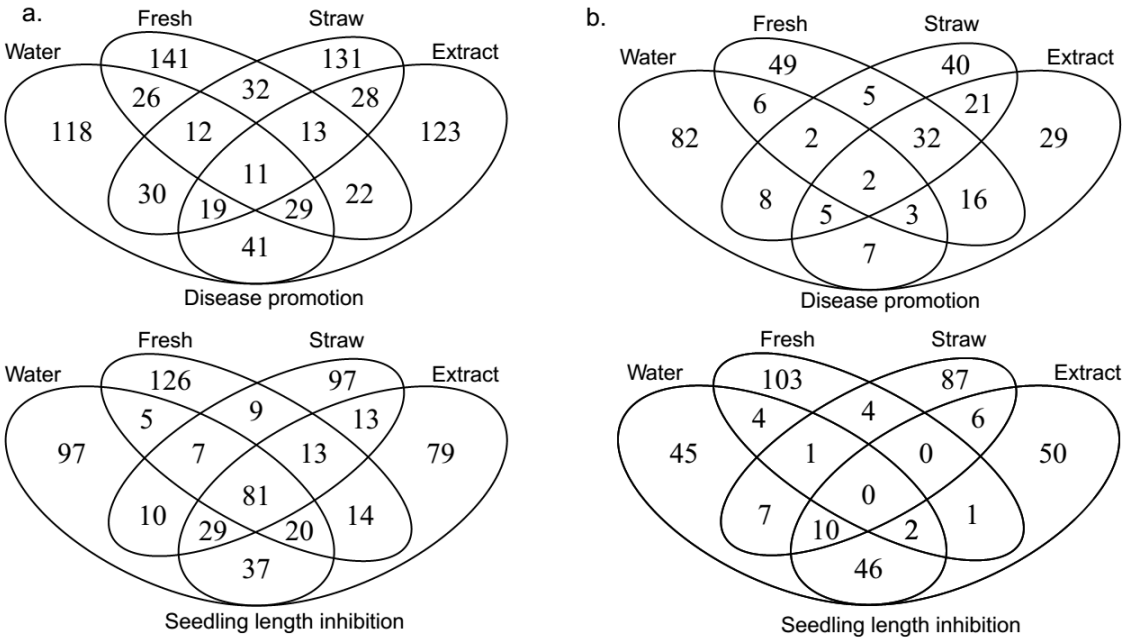

Figure B.1. Venn diagram shows the shared and unique (a) bacterial and (b) fungal OTUs with weed suppressive roles from water, water-soluble extracts, fresh residues, and straw residues treatments.

APPENDIX C: SUPPLEMENTARY INFORMATION FOR CHAPTER 4

Table C.1. Percentage of shared OTUs between different fields in the same management system and fields in different management systems.

	Microbial species	Same management system (Between two fields)	Different management system
Enriched on dead seeds	Bacteria	26%+5.4%	12%+2.4%
	Fungi	32%+2.3%	18%+2.1%
Enriched on diseased live seedlings	Bacteria	13%+2.7%	5.9%+1.4%
	Fungi	17%+2%	4.5%+1.6%
Responded to addition of fresh residues	Bacteria	39%$\pm$3.7%	31%+2.4%
	Fungi	37%$\pm$5.6%	35%+2.3%

Table C.2. Putative weed suppressive microbes that were identified both in Chapter 3 and this study. Top ten most frequently identified bacterial and fungal OTUs in two studies were shown.

OTU	Domain	Phylum	Class	Order	Family	Genus	Species	Eriched by residues
175	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium		This study
68	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus		Not enrich
165	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	Clostridium		This study
17	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadacea			Both studies
3	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriale	Enterobacteriacea			Both studies
31	Bacteria	Firmicutes	Bacilli	Bacillales	Paenibacillaceae	Paenibacillus		This study
4599	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oalobacteraceae	Massilia	Massiliatimona	Not enrich
4965	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadale	Pseudomonadace	Pseudomonas		Both studies
5	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiaceae	Agrobacterium		Both studies
2	Bacteria	Proteobacteria	Gammaproteobacteria	anthomonadales	anthomonadaceae	Stenotrophomona		This study
394	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadale	Pseudomonadace	Pseudomonas		Both studies
2	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium		Chapter 3
103	Fungi	Zygomycota	Incertaesedis	Mucorales	Mucoraceae	Mucor		Chapter 3
7	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae			Not enrich
3470	Fungi	Zygomycota	Incertaesedis	Mucorales	Mucoraceae	Actinomucor	Actinomucorel	Chapter 3
527	Fungi	Unassigned	Unassigned					Not enrich
129	Fungi	Unassigned	Unassigned					Chapter 3
17	Fungi	Ascomycota	Sordariomycetes	Sordariales	Lasiosphaeriacea	unidentified		Not enrich
18	Fungi	Basidiomycot	Agaricomycetes	Cantharellales	Ceratobasidiaceae	unidentified		Chapter 3
2374	Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	Fusarium		This study
52	Fungi	Zygomycota	Incertaesedis	Mortierellales	Mortierellaceae	Mortierella	Mortierellaamb	Chapter 3

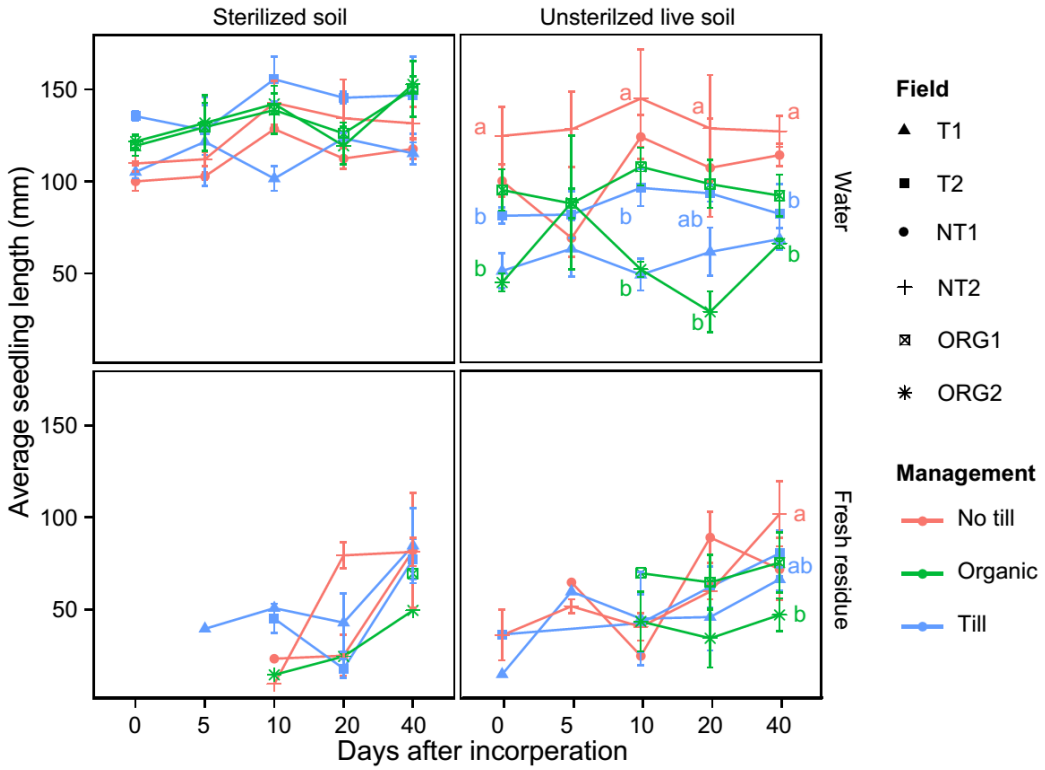

Figure C.1. Seedling length of mustard with water or fresh residues in sterilized soil and live soil. Standard error from three replicate analyses are shown. Letter a-b indicate significant difference among fields at P < 0.05 by a Tukey's HSD test. The color of letter indicates the management system.

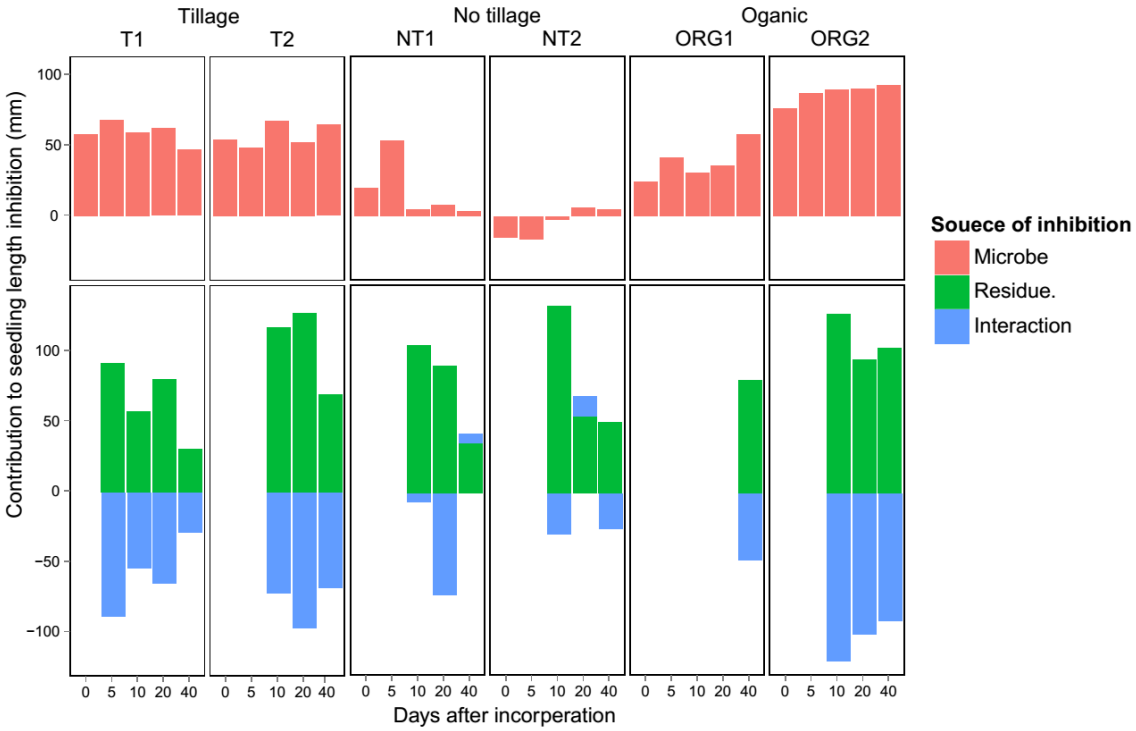

Figure C.2. Antagonistic interactions between soil microorganisms and red clover residues differ between agricultural systems. Bars indicate the strength of microbe-only, residue-only, and microbe by-residue contributions to seedling growth inhibition.

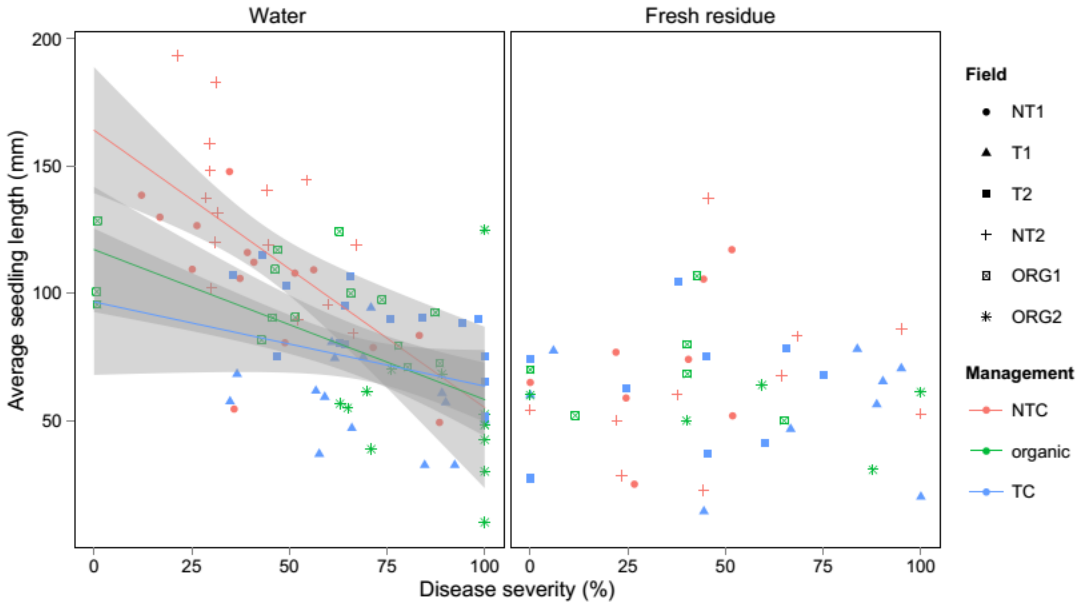

Figure C.3. Effects of diseased severity of seedling for treatments exposed to water and fresh residues. Disease severity: the length of visible necrotic lesions divided by the total length of germinated seedlings.

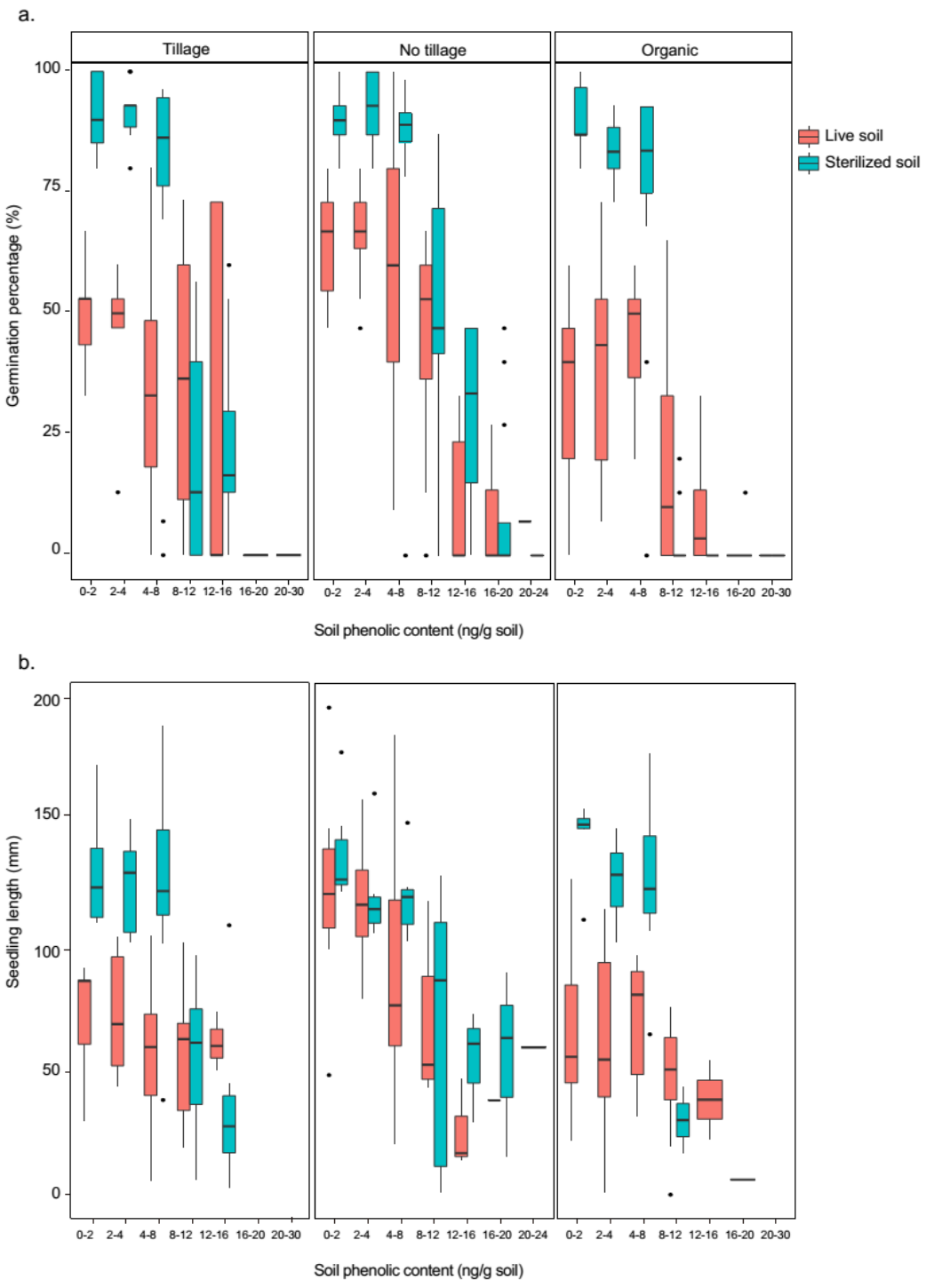

Figure C.4. Effects of soil phenolic content on the (a) germination percentage and (b) seedling length for treatments exposed to water and fresh residues in three agricultural systems.

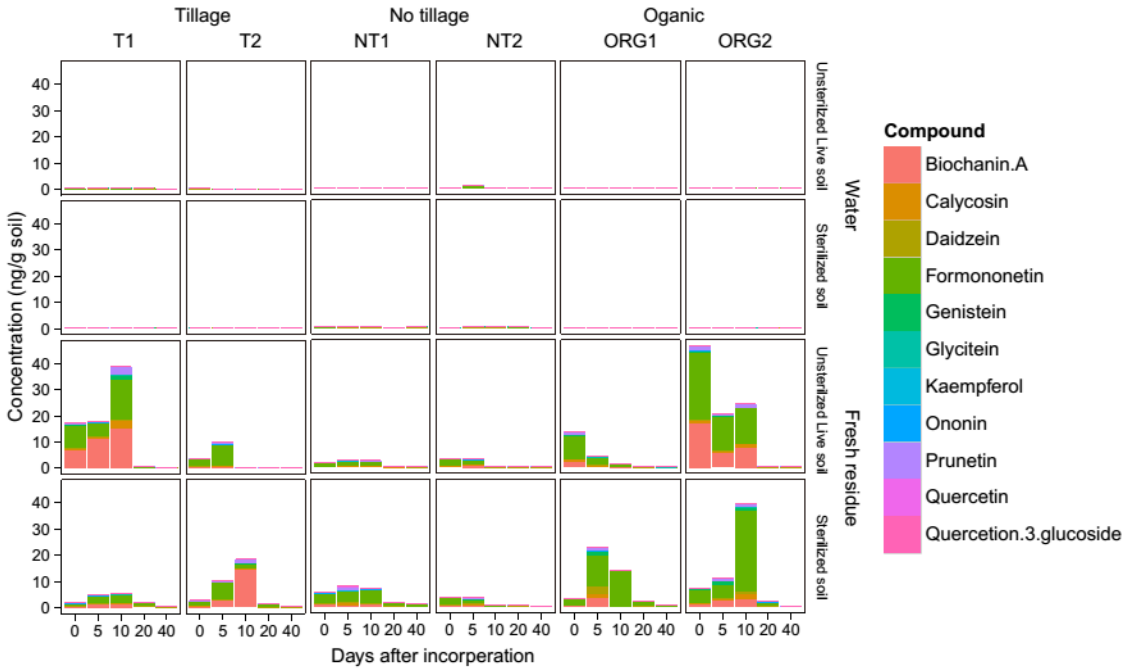

Figure C.5. Phenolic compounds in sterile and live soil amended with water or fresh residues.

APPENDIX D: SUPPLEMENTARY INFORMATION FOR CHAPTER 5

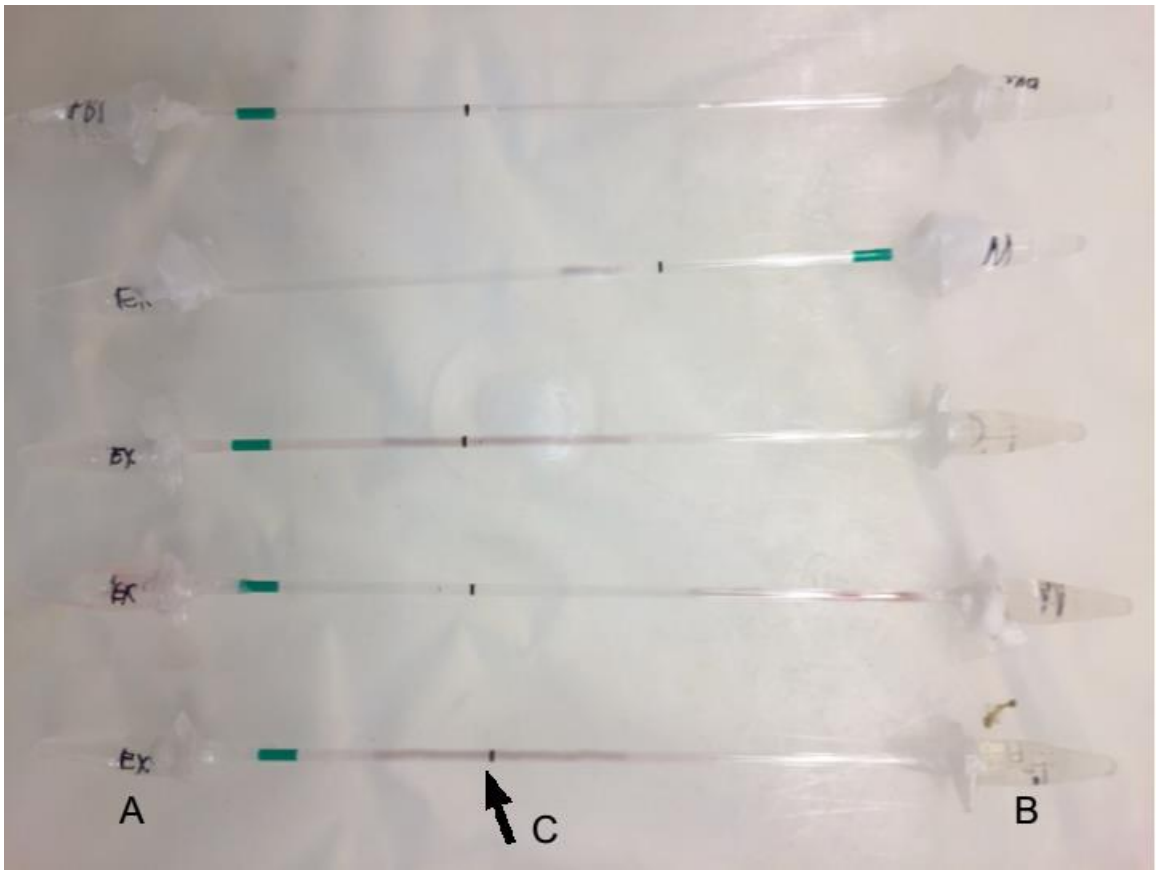

Figure D.1. Chemotaxis of soil microbial suspension in microcapillary tubes with root exudates or control solutions traced by reaction with tetrazolium. The right end of the micro-capillary tube (A) was inserted into another 0.2 ml tube containing either the water soluble root exudates or control solution (phosphate buffer, Hoagland's solution or water). The left side of a microcapillary tube was placed in a suspension of soil microbial (or water as control) present in a 0.2 ml eppendorf tube (B). Movements of microbes were visualized by tetrazolium (red color) (C).

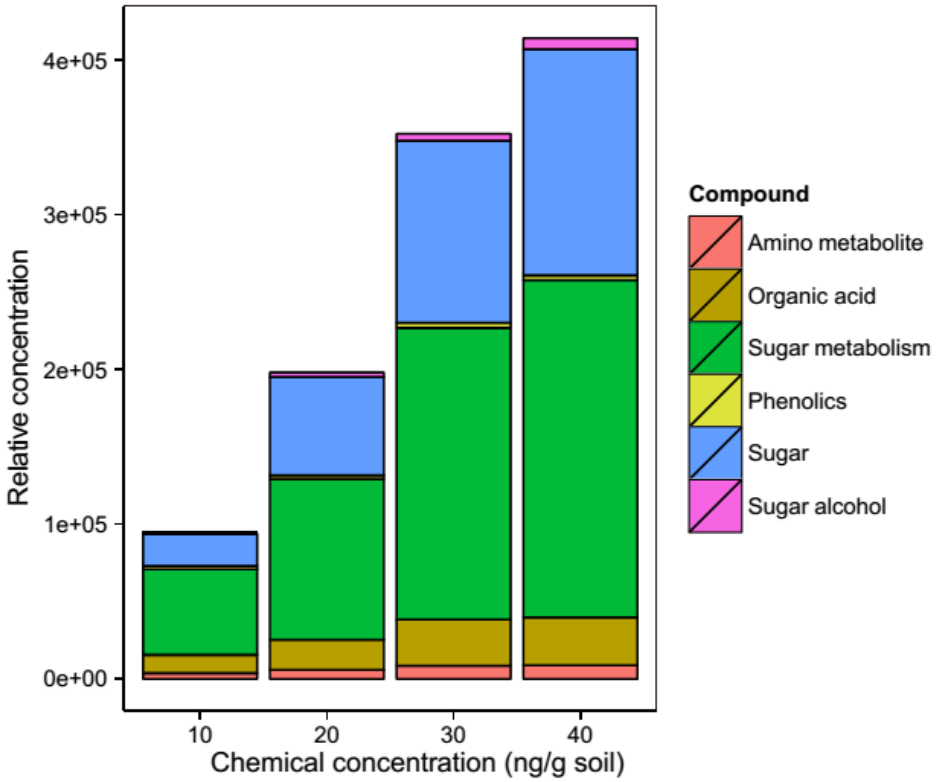

Figure D.2. Relative concentrations of top five metabolites from water extract of red clover residue. Concentrations of residue water extract are target concentrations of soil phenolics.